

Pathogens in free-ranging African carnivores: evolution, diversity and co-infection

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SUMMARY

The ecological role of most wildlife pathogens is poorly understood because pathogens are rarely studied in relation to the long-term population dynamics of wildlife hosts. Instead, pathogen infections are reported on a case basis or studies are focused on periods when pathogens cause noticeable mortality in their hosts. However, pathogens that appear to be of low virulence may also have an important effect if they operate in a synergistic fashion or affect life history parameters such as longevity or reproductive success. Furthermore, the effect of pathogens on population dynamics may be difficult to detect in wildlife, for example if they reduce the survival of young age classes that are rarely observed. Until now, research on the life history consequences of pathogen infection has mainly been confined to laboratory studies where animals are raised and kept under strictly defined conditions, or to small, short lived species such as rodents, birds or insects, as well as to human populations.

The aim of this thesis was to address these problems by assessing the impact of single infections and co-infections by pathogens on key life history parameters and the influence of life history traits on infection status in a free-ranging social carnivore species, the spotted hyena *Crocuta crocuta*. The study was embedded in a long-term study on several clans of spotted hyenas from two subpopulations inhabiting the Serengeti National Park and the adjacent Ngorongoro Crater in Tanzania, East Africa. Data on key life history parameters were available for several hundred individually known animals as was information on changing levels of prey availability. I established molecular biological methods (polymerase chain reactions (PCRs) and reverse transcription PCRs) to screen an extensive set of faecal, blood and tissue samples from individually known spotted hyenas and sympatric carnivores for the presence of coronavirus, calicivirus, canine distemper virus, canine parvovirus and the tick-borne blood parasite *Hepatozoon* sp. to determine the prevalence of the pathogens.

Phylogenetic analyses revealed several new variants of pathogens in spotted hyenas and other sympatric carnivore species. I detected previously undescribed coronavirus variants and an unexpected high diversity and rapid temporal change of coronavirus variants circulating in one spotted hyena subpopulation across different years (Chapter 3.1); a result of considerable relevance to our understanding of coronaviral infections in wildlife populations. These results also highlight the importance of long-term monitoring of viral populations in wildlife.

I identified previously undescribed calicivirus-like variants in spotted hyenas which were more closely related to human sapoviruses than to those typically infecting carnivore species

(Chapter 3.2). This result suggests a host species jump from humans to spotted hyenas followed by adaptation of the pathogen to the new host.

I identified age-specific patterns of single infections with coronavirus or calicivirus whereby young Serengeti hyenas were significantly more likely to excrete virus than adults (Chapter 3.1, 3.2). To assess the possible effects of key life history parameters on the likelihood of infection with coronavirus, calicivirus, or both viruses, I applied a statistical model which included concurrent infection with helminths (Chapter 3.3). This model revealed that the most important factors influencing the likelihood of viral infection was (1) simultaneous helminth infection, suggesting that modulation of the immune response to helminth infection reduced the chance of virus infection, and (2) the synergistic effects of nutritional and physiological stress induced by low prey availability, thereby reducing maternal milk support which in turn is likely to lead to increased sibling rivalry in twin litters. Additionally, I investigated whether infection status as a juvenile reduced longevity and found that there was evidence of natural selection by non-virulent pathogens on a host population (Chapter 3.3). The longevity differed between individuals with either a single coronavirus- or calicivirus infection or individuals co-infected with both viruses. A single infection with coronavirus had the worst impact whereas co-infection with both viruses had more benign consequences. These results showed that key ecological parameters may influence infection status of juvenile spotted hyenas and that infection status in turn may significantly influence longevity.

Furthermore, my research revealed evidence of coronavirus infection in the Serengeti hyena population and a lack of current infection in the Ngorongoro Crater population (Chapter 3.4). This difference in viral presence between the two subpopulations was probably caused by a significantly higher persistence of susceptible individuals in clans in the Serengeti subpopulation than in the Crater subpopulation, even though both subpopulations were part of the same metapopulation and shared the same social structure. This result illustrates that pathogen dynamics within apparently similar and closely adjacent subpopulations may significantly differ as a consequence of variation in demographic stochasticity.

My phylogenetic analysis on *Hepatozoon* sp. revealed variants that infect sympatric carnivore hosts (Chapter 3.5). Monitoring of young spotted hyenas less than two months of age showed that *Hepatozoon* infections, previously thought to be benign in this species, cause mortality in young animals.

I identified canine distemper virus (CDV) infection as cause of mortality in an African wild dog *Lycaon pictus* pack close to the Serengeti National Park boundary that was not associated

with increased CDV mortality among wild carnivores inside the Park (Chapter 3.6). The phylogenetic analysis of this variant provided further information on the diversity of CDV variants infecting wild carnivores, and evidence of different CDV variants adapted to canids in comparison to variants in lions and spotted hyenas. Thus the variant I identified in wild dogs will be of key importance to future research on CDV in the wild carnivore guild in this world biodiversity site.

This is the first study that combines ecological and epidemiological data from a long-term study on a host with extensive molecular genetic and phylogenetic studies on a variety of pathogens to investigate the impact of single infections and co-infections by pathogens on key life history parameters and the influence of life history parameters on infection status in a free-ranging social mammal species, the spotted hyena.

Keywords:

African carnivores, pathogens, co-infection, spotted hyena, life histories

ZUSAMMENFASSUNG

Die ökologische Rolle der meisten Wildtier-Pathogene in Bezug zur langfristigen Populationsdynamik ihrer Wirte ist nur ansatzweise erforscht und wird deshalb nur unzureichend verstanden. Stattdessen ist die Erforschung von Infektionen mit Pathogenen oft beschränkt auf einzelne Fallstudien oder auf Perioden mit deutlich erhöhten Mortalitätsraten innerhalb der Wirtspopulation. Pathogene mit geringer Virulenz können jedoch durch synergistisches Auftreten verheerende Auswirkungen auf die Fitness ihrer Wirte haben oder sich auf wichtige individuelle lebensgeschichtliche Merkmale, wie zum Beispiel Lebensdauer oder Reproduktionserfolg, auswirken. Des Weiteren sind die Auswirkungen von Krankheitserregern auf die Populationsdynamik der Wildtiere schwer abzuschätzen, zum Beispiel wenn sie die Überlebenschancen von selten zu beobachtenden Jungtieren beeinträchtigen. Bis heute sind Untersuchungen von Infektionen mit Pathogenen und deren Auswirkungen auf Lebensgeschichten meist auf Laborstudien beschränkt, in denen Tiere unter streng definierten Bedingungen gezüchtet und gehalten werden, bzw. auf Studien an kleinen, kurzlebigen Arten wie Nagern, Vögeln und Insekten oder auf Studien an der Humanpopulation.

Ziel dieser Arbeit war, die Auswirkungen von Einzel- oder Koinfektionen auf individuelle lebensgeschichtliche Schlüsselparameter sowie die Einflüsse bestimmter lebensgeschichtlicher Merkmale auf den Infektionsstatus anhand einer frei lebenden sozialen Karnivorenart, der Tüpfelhyäne *Crocuta crocuta*, zu untersuchen. Diese Arbeit war in eine Langzeitstudie integriert und wurde an mehreren Gruppen von Tüpfelhyänen zweier Subpopulationen durchgeführt, die im Serengeti Nationalpark sowie in dem angrenzenden Ngorongoro Krater in Tansania in Ostafrika lebten. Daten über wichtige lebensgeschichtliche Merkmale von mehreren hundert individuell bekannten Tieren in Kombination mit Daten über die wechselhafte Beuteverfügbarkeit in den Territorien der Gruppen standen hierfür zur Verfügung. Ich etablierte molekularbiologische Methoden (Polymerase Kettenreaktionen (PCRs) und Reverse Transkriptions PCRs), um eine Vielzahl an Kot-, Blut- und Gewebeproben individueller Tüpfelhyänen und sympatrisch lebender Karnivoren auf das Vorkommen von Coronaviren, Caliciviren, Hundestaupe-Viren, Parvoviren sowie eines von Zecken übertragenen Blutparasiten, *Hepatozoon* sp., zu untersuchen.

Meine phylogenetischen Untersuchungen brachten verschiedene neue Pathogenvarianten in Tüpfelhyänen und anderen sympatrisch lebenden Karnivoren hervor. Ich entdeckte bisher unbeschriebene Coronavirus Varianten, die eine unerwartet hohe Diversität sowie eine rasche Anpassungsfähigkeit innerhalb weniger Jahre in der Tüpfelhyänenpopulation in der Serengeti

(Kapitel 3.1) aufwiesen; ein Ergebnis welches beträchtlich zum Verständnis der Coronavirus Varianten, die in der Wirtspopulation zirkulieren, beiträgt. Diese Resultate heben die Wichtigkeit von Langzeitstudien an Viruspopulationen in Wildtieren hervor.

Ich identifizierte bisher unbeschriebene Calicivirus-ähnliche Varianten in Tüpfelhyänen, welche näher mit humanen Sapoviren verwandt waren als mit Caliciviren, die üblicherweise Karnivoren infizieren (Kapitel 3.2). Dieses Resultat deutet auf eine Übertragung von Mensch auf Tüpfelhyäne hin, woran sich eine Anpassung an den neuen Wirt angeschlossen hat. Ich konnte altersspezifische Muster bei Einzelinfektionen mit Corona- und Caliciviren nachweisen, wobei Jungtiere häufiger Viren ausschieden als adulte Hyänen (Kapitel 3.1, 3.2). Um den potentiellen Einfluss bestimmter lebensgeschichtlicher Schlüsselparameter auf Einzelinfektionen mit Coronaviren, Caliciviren, oder Koinfektionen mit beiden Viren zu untersuchen, wandte ich ein statistisches Model an, das eine gleichzeitige Infektion mit Helminthen einschloß (Kapitel 3.3). Dieses Model ergab, dass die wichtigsten, die Wahrscheinlichkeit der viralen Infektion beeinflussenden, Faktoren folgende waren: (1) simultane Infektionen mit Helminthen, was darauf hinweisen könnte, dass eine von Helminthen hervorgerufene Modulation der Immunantwort, die Gefahr einer viralen Infektion reduziert, und (2) synergistische Effekte von physiologischem Stress und Nahrungsmangel, begründet durch geringe Beuteverfügbarkeit und damit assoziierter Unterversorgung der Jungtiere mit Muttermilch, was wiederum zu gesteigerter Geschwisterrivalität in Zwillingswürfen geführt haben könnte. Zusätzlich untersuchte ich, ob der Infektionsstatus im Jungtieralter die Lebensdauer reduzierte und fand Hinweise, dass natürliche Selektion innerhalb der Wirtspopulation durch nicht-virulente Erreger stattfand (Kapitel 3.3). Die Lebensdauer unterschied sich zwischen Individuen, die entweder mit Corona- oder Caliciviren infiziert waren, und Individuen, die mit beiden Viren koinfiziert waren. Einzelinfektionen mit Coronaviren hatten schwerwiegendere Folgen, wohingegen Koinfektionen mit beiden Viren weniger gravierende Auswirkungen hatten. Diese Ergebnisse veranschaulichen, dass ökologische Schlüsselparamter den Infektionsstatus von jungen Tüpfelhyänen, und umgekehrt der Infektionsstatus die Lebensdauer, signifikant beeinflussen könnte.

Darüber Hinaus konnte ich in der Hyänensubpopulation in der Serengeti Coronaviren nachweisen, wohingegen in der Subpopulation im Ngorongoro Krater keine nachweisbar waren (Kapitel 3.4). Das unterschiedliche Vorkommen von Coronaviren in den beiden Subpopulationen, welche Teil einer Metapopulation waren und eine identische Sozialstruktur aufwiesen, beruhte möglicherweise auf das signifikant höhere Vorhandensein anfälliger Individuen in den Gruppen in der Serengeti Subpopulation im Gegensatz zur Krater Subpopulation. Dies

verdeutlicht, dass sich die Pathogendynamik aufgrund demographischer Stochastizität in augenscheinlich gleichartig strukturierten und aneinander angrenzenden Subpopulationen signifikant unterscheiden kann.

Meine phylogenetischen Untersuchungen von *Hepatozoon* sp. ergaben, dass verschiedene Varianten unterschiedliche Karnivorenarten infizieren können (Kapitel 3.5). Beobachtungen an Tüpfelhyänen, jünger als zwei Monate, zeigten, dass Infektionen mit *Hepatozoon*, welche in dieser Tierart bisher als mild beschrieben wurden, auch tödlich verlaufen können.

Ich identifizierte Infektion mit Hundestaupavirus (CDV) als Todesursache in einem Rudel Afrikanischer Wildhunde *Lycaon pictus*, nahe der Grenze zum Serengeti Nationalpark, wobei kein Zusammenhang mit einer erhöhten CDV Mortalität bei wilden Karnivoren innerhalb des Parks festgestellt werden konnte (Kapitel 3.6). Die phylogenetische Analyse lieferte zusätzliche Informationen über die Diversität der CDV Varianten, welche wildlebende Karnivoren infizieren, sowie Hinweise auf Anpassung bestimmter CDV Varianten an Hundartige, im Vergleich zu Löwen und Hyänen. Diese in Wildhunden identifizierte Variante wird für die künftige CDV Forschung in der wild lebenden Karnivoren-Gemeinschaft dieses Biodiversitäts-Areals von großer Bedeutung sein.

Dies ist die erste Studie an einer Wirtspopulation, in welcher ökologische und epidemiologische Daten einer Langzeitstudie mit umfangreichen molekulargenetischen und phylogenetischen Untersuchungen einer Vielzahl von Pathogenen kombiniert wurden, um den Einfluss von Einzel- und Koinfektionen auf lebensgeschichtliche Schlüsselmerkmale, sowie den Einfluss lebensgeschichtlicher Merkmale auf den Infektionsstatus in einer frei lebenden sozialen Säugetierart, der Tüpfelhyäne, zu untersuchen.

Schlagwörter:

Afrikanische Karnivoren, Pathogene, Koinfektion, Tüpfelhyäne, Lebensgeschichten

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1 GENERAL INTRODUCTION

The ecological role of most wildlife pathogens is poorly understood because pathogens are rarely studied in relation to the long-term population dynamics of wildlife hosts (Grenfell & Dobson 1995, Tompkins & Begon 1999). Instead, pathogen infections are reported on a case basis or research is focused on periods when pathogens cause noticeable mortality in their hosts. However, pathogens that cause moderate or low levels of mortality may also have an important effect if they operate in a synergistic fashion (Graham et al. 2007; Munson et al. 2008), or affect life history parameters such as reproduction or longevity (Thabti et al. 2002; Vilcek & Nettleton 2006). To date, field studies on the relationship of infection status and life history parameters in natural populations are scarce and those studies that exist often fail to investigate the possible relationship between the presence of an infection during one life history stage and the manifestation of the effect of infection at a later stage. Furthermore, few studies have considered the impact of simultaneous infection with several pathogens on individuals, even though such multiple infections are likely to be a frequent occurrence in wildlife populations. Although the effect of various intrinsic and extrinsic factors on an individual's ability to respond to infection has been investigated in laboratory experiments and humans, far less is known about the effects of either intrinsic or extrinsic factors, and potential interactions between these factors on the susceptibility of individuals in free-ranging wildlife populations to pathogen infection. This complexity presents a considerable challenge to those seeking to unravel the true effect of pathogens on free-ranging wildlife populations (East et al. in press). This challenge has to be met, particularly when assessing the impact of pathogens on vulnerable, rare or endangered species or populations.

Simultaneous infection

The ability of individuals within a population to effectively combat infections might vary because the level of virulence of a particular pathogen may be altered by the presence or absence of other pathogens. For example, immunosuppression owing to the presence of human immunodeficiency virus (HIV) has contributed to the spread of numerous secondary infections such as the fungal pathogen *Pneumocystis carinii* and the *Mycobacterium tuberculosis* and influences the clinical consequences of secondary infections in humans (Kovacs et al. 1984; Idemyor 2007). Another example in free-ranging lions was found by Munson et al. (2008) who suggested that single infections with canine distemper virus (CDV) in lions *Panthera leo* did not cause clinical cases but co-infection with the tick-borne pathogen *Babesia* sp. resulted in an epidemic.

Intrinsic factors

Intrinsic factors that influence an individual's vulnerability to infection include for example genotype, age, nutritional status and reproductive status.

Genetic factors have been shown to influence an individual's susceptibility to infection, most notably in relation to genes linked to immune function such as the major histocompatibility complex (MHC) which is a part of the genome involved in the presentation of antigens to the vertebrate immune system (e.g. Jepson et al. 1997). For example, an impact of genetic variation on macroparasite resistance was observed in a feral Soay sheep population *Ovis aries* on the Scottish Island St. Kilda where genetic variation was associated with different levels of resistance to a nematode (Gulland et al. 1993; Paterson et al. 1998). There is evidence that the loss of genetic heterozygosity – often a consequence of inbreeding in small and isolated populations – might result in a reduction in resistance to parasites (Crnokrak & Roff 1999).

Age is another important intrinsic factor that is likely to influence not only an individual's susceptibility to infection but also the severity of the disease once infection has occurred (Beldomenico et al. 2008; Alonso-Alvarez et al. 2010). In general, young individuals with a naïve or not yet fully developed immunesystem are more prone to infection and disease outcome may be more severe than in older animals. Ross et al. (1993) for example found that in pups of the harbour seal *Phoca vitulina*, lymphocyte function and total immunoglobulin G levels were low at birth and increased when approaching weaning. But also young animals postweaning might be at higher risk of infection probably as a result of not receiving protective maternal antibodies through milk from their mothers anymore. Wang et al. 2006 for example observed that prevalence of infection with a porcine sapovirus was higher in postweaning pigs than in nursed piglets and adult pigs probably due to not receiving maternal antibodies anymore. In Chapter 3.1 I will demonstrate that the prevalence of coronavirus infection is significantly higher in spotted hyena *Crocuta crocuta* cubs than in adult animals, and together with colleagues I have shown that young spotted hyenas infected with a blood parasite (*Hepatozoon* sp.) that is benign in adult animals can cause mortality in young cubs (East et al. 2008).

Nutrient limitations can lead to reduced immunocompetence (Gershoff et al. 1968; Jose & Good 1973; Gross & Newberne 1980) and thus increase susceptibility to infectious diseases (e.g. Piyathilake et al. 2004). In humans malnutrition associated with bacterial, viral or protozoal agents are considered as a major risk factor for morbidity and mortality worldwide (Scrimshaw & SanGiovanni 1997, Ambrus & Ambrus 2004). Evidence of associations be-

tween poor nutritional status and an increase in the probability of acquiring infection with pathogens and decreased survival as result in reports from wildlife is scarce (Beldomenico et al. 2008).

Furthermore, when inadequate body resources are available, investment in immune function may be curtailed if body resources are allocated to other important functions such as growth or reproduction (Bonneaud et al. 2003; Piyathilake et al. 2004). Reproduction is an intrinsic factor that is costly in terms of body resources and is thus likely to influence the ability of animals to maintain adequate immune function. The immunological study by Ross et al. (1993) on common seals revealed that lymphocyte function and total immunoglobulin G levels were reduced in mothers at the end of lactation and an impaired ability to invest in immune function probably explains why Plowright et al. (2008) found that pregnant and lactating little red flying foxes *Pteropus scapulatus* had a higher risk of infection with hendra virus than non-breeding females.

Extrinsic factors

Extrinsic factors include for example food supply, seasonal changes in day length and climate, population densities and social stress.

In temperate regions, several extrinsic factors associated with winter, including declining temperatures, food supply and day length, are known to result in a decline in immune function. Recently Gasparini et al. (2006) observed a seasonal decline in humoral immunocompetence in kittiwakes *Rissa tridactyla* that could be explained by variation in food supply throughout the breeding season. Anthropogenic factors such as pollution, habitat fragmentation and global climate change are a cause for concern because of their potential to directly decrease immune function (as is the case with many pollutants) or to indirectly alter allocation of resources to immunity by decreasing access to food resources (as is the case with habitat fragmentation and some aspects of global climate change).

Global climate change may favour the transmission of pathogens, particularly those spread by vectors favoured by changing climatic conditions. The mosquitoes *Aedes albopictus* and *Aedes aegypti* are the primary vectors for arboviral pathogens such as dengue virus, yellow fever virus and chikungunyavirus (Gould & Higgs 2009) that cause a variety of diseases in humans. Global climate change is predicted to permit these vectors to disperse beyond their current geographic boundaries, leading to more cases of epidemic outbreaks in Europe (Gould & Higgs 2009; Lafferty et al. 2009a,b). Even small climatic changes can dramatically amplify mosquito abundance, and in highland areas with relatively low mosquito numbers, even small

increases in mosquito abundance can significantly increase malaria transmission (Pascual et al. 2006, 2009).

Host population density and contact rates between susceptible and infected individuals, within and between populations or groups of individuals, also plays an important role in pathogen transmission (Anderson & May 1991; Diekmann & Heesterbeek 2000; Lindholm & Britton 2007; Webb et al. 2007). Pathogen transmission usually increases with host density and contact rates and declines with a reduction in the number of hosts and contact between susceptible and infected individuals (Anderson & May 1991; Diekmann & Heesterbeek 2000; Hudson et al. 2002).

There is growing evidence that social status in many social mammalian species, including humans, is an extrinsic factor that has wide-ranging effects on the health status of individuals and their susceptibility to infections (Sapolsky 2005). As high social status typically provides priority of access to food resources, top ranking individuals and their offspring generally have a better nutritional status than subordinates (Golla et al. 1999; Hofer & East 2003, 2008) and are thus more likely to allocate sufficient body resources to immune function than animals at the bottom of the hierarchy. Animals living in social societies are uninevitably exposed to social stress and chronic exposure to 'stress' in social mammalian species, for example in spotted hyenas during periods of hierarchy formation or social instability, can result in elevated levels of glucocorticoids as a physiological 'stress' response (Sapolsky 1982; Saltzman et al. 1994; Goymann et al. 2001). This can result in negative physiological and pathological consequences, one of which is an impairment of the immune system (Toates 1995). Such a reduction in components of the active immune system is likely to result in an increased susceptibility to pathogen infection, morbidity or even mortality (Hofer & East 1998).

Although pathogen infection may be influenced by either intrinsic or extrinsic factors, interactions between various combinations of intrinsic and extrinsic factors are probably common but difficult to quantify.

Pathogen emergence and evolution

The evolutionary potential of pathogens may have an impact on their virulence. Novel pathogens can emerge through mutations or recombination events and might have a negative impact on host populations since they are often more virulent than the original variants from which they emerge (Daszak et al. 2000). Pathogens that jump from one host species to another may also result in the emergence of novel infectious diseases. Such species jumps have resulted in fatal disease epidemics. This is illustrated by the case of the human disease AIDS

(acquired immunodeficiency syndrome) caused by HIV, thought to have evolved from the non-human primate simian immunodeficiency virus (SIV) that jumped to humans and then spread through the human population worldwide as a human HIV pandemic (Hahn et al. 2000). Another example is the highly pathogenic avian influenza virus H5N1 which is capable of being transmitted from animal reservoir species to humans (Kuiken et al. 2006). A wildlife example of a virus jumping to a new host species is the transmission of phocine distemper virus (PDV) from what is thought to be its original host, the harp seal *Pagophilus groenlandicus*, to the European population of the harbour seal which resulted in two recent large scale epidemics and mass mortality in harbour seal populations around the North Sea in 1988 and 2002 (Hudson et al. 2002).

In principle there are three processes that are preconditions for a virus to cross the host species barrier, and the likelihood of a virus becoming endemic in a new host species depends on the existence of these processes (Woolhouse et al. 2005; Nieberding & Olivieri 2007; Davies & Pedersen 2008). Firstly, there must be interspecific interactions between virus donor host and recipient species that enable the transmission of the virus between the two. Secondly, following contact with the virus transmitted from a donor host, interactions should occur between the recipient host species and the virus. Finally, the virus should be able to be transmitted at a sufficient rate between members of the new recipient host species during host-host interactions. For any type of transmission, there must be sufficient contact between donor and recipient species and enough compatibility between the virus and the new host to allow replication and the possibility of transmission to other members of the recipient species (Kuiken et al. 2006). The expected impact of a pathogen outbreak on a host species or population depends on the transmission potential of a pathogen from one host individual to another or from a population in which the pathogen occurs to a vulnerable, naïve population of the same host species.

In recent years mathematical models have become an important tool for unravelling the epidemiology of infectious pathogens, by promoting increased understanding of pathogen transmission, persistence and extinction (Anderson & May 1979a,b, see also 1991; Grenfell & Dobson 1995; Diekmann & Heesterbeek 2000). Recent models are more realistic as they facilitate the inclusion of heterogeneity of host individuals within populations, by dividing host populations into sub-communities that experience different levels of social interaction and pathogen transmission (Anderson & May 1985, 1991; Hagenaars et al. 2004; Lindholm & Britton 2007). It has also been recognised that the dynamics of viral pathogens and the ability of an infectious pathogen to persist in a population depends on several factors, including the

transmission characteristics of the pathogen, host social structure, host age and variation in the immunocompetence of individual hosts (Anderson & May 1991; Lindholm & Britton 2007). When contact between infectious and susceptible individuals is high, pathogens may become extinct if the period that host individuals are infectious is relatively brief and infection induces life-long immunity to re-infection. Infectious pathogens that are endemically transmitted in their host population may experience extinction when the chain of transmission terminates or is interrupted.

Basic aims of this study

The basic aims of my research were to: (1) develop non-invasive molecular biological screening techniques that would permit me to identify pathogen infections in individually known spotted hyenas, belonging to two subpopulations of a hyena metapopulation, and sympatric carnivores in the Serengeti ecosystem in Tanzania, East Africa; (2) determine the genetic diversity and phylogenetic relationship of pathogens present in these two subpopulations in relation to other sympatric carnivore species in this ecosystem and other geographical regions; (3) determine infection patterns and prevalence of these pathogens in the two hyena subpopulations; (4) investigate which life history, social and ecological factors influence susceptibility to single and concurrent infections and to assess the impact of single and multiple infections on key life history parameters.

My research was part of a long-term research programme on two well habituated spotted hyena subpopulations in the Serengeti National Park and the adjacent Ngorongoro Crater, Tanzania. Information on life history and ecological parameters such as age, social status, sex, longevity, prey availability and nutritional status of individually known hyenas was available as well as an extensive set of faecal samples plus blood and tissue samples from anaesthetised animals or animals necropsied after they were found dead in the field. The Serengeti spotted hyena was a suitable model host species for my research aims, since it is the most abundant large carnivore species in both the Serengeti National Park (Hofer & East 1995) and Ngorongoro Crater (Höner et al. 2001, 2005) and a highly social species that can be easily observed at social centres such as the clan communal den areas inside clan territories (Hofer & East 1993), from which faecal samples for various analyses can be readily obtained (Goymann et al. 2001; East et al. 2003, 2004; Höner et al. 2007). Spotted hyena society is female-dominated and structured by separate female and immigrant male linear dominance hierarchies (East & Hofer 2001). As a result of rank-related access to food resources in clan territories, the nutritional status of adult females and their offspring is closely associated with social status (Golla et al. 1999; Hofer & East 2003). High-ranking females are preferred social part-

ners (East et al. 1993; Burgener et al. 2009) and thus social status has a significant impact on contact rates and the transmission of infectious pathogens among group members (East et al. 2001). Furthermore, as all dependent offspring in a social group are raised together in a clan's communal den, it is possible to monitor the health status of young animals (East et al. 2008) and to obtain faecal samples from young when they use communal latrines in the vicinity of the den (East et al. 2004).

The Serengeti ecosystem is defined as the area covered by the migratory movements of herbivores (Sinclair & Arcese 1995). Although there is some movement of migratory herbivores in and out of the adjacent Ngorongoro Crater, wildlife populations on the Crater floor are mostly resident and are separated from the southern short grass plains of the Serengeti ecosystem by the wall of the Ngorongoro caldera (Runyoro et al. 1995). An investigation of mitochondrial (mt) DNA haplotypes from spotted hyenas in the Ngorongoro Crater and Serengeti National Park demonstrate the presence of a distinct haplotype in the Crater (Albert et al. 2000). The Serengeti ecosystem has a species-rich community of 26 carnivore species (Sinclair & Arcese 1995) that include large populations of several species belonging to the families Canidae, Felidae, Hyaenidae, Viverridae and Herpestidae (Hofer & East 1995) that might serve as reservoirs for pathogens transmitted by multiple carnivore species such as canine distemper virus (CDV) (Haas et al. 1996; Roelke-Parker et al. 1996; Carpenter et al. 1998).

Pathogens infecting carnivores in the Serengeti ecosystem have been studied since the 1960s. Serengeti spotted hyenas and their closest ecological competitors, lions and cheetahs *Acinonyx jubatus* are known to be exposed to tick-borne pathogens of the genera *Babesia* sp., *Theileria* sp. and *Hepatozoon* sp. (Averbeck et al. 1990; Peirce et al. 1995; Packer et al. 1999; East et al. 2008) and have been exposed to viruses such as rabies, CDV and coronavirus (CoV) (Schaller 1972; Burrows et al. 1994; Hofer & East 1995; Haas et al. 1996; Hofmann-Lehmann et al. 1996; Roelke-Parker et al. 1996; East et al. 2001, 2004; Harrison et al. 2004). A serological survey on spotted hyenas in the Maasai Mara National Reserve, part of the Serengeti ecosystem and located adjacent to the Serengeti National Park, demonstrated that spotted hyenas had been infected with and mounted immune responses to feline and canine viruses including feline immunodeficiency virus (FIV), feline calicivirus (FCV), feline panleukopenia virus/canine parvovirus (FPV/CPV), feline herpesvirus 1 (FHV1) and CDV (Harrison et al. 2004).

However, the molecular genetics and distribution for only a few pathogens have been studied in various host species in the Serengeti ecosystem, with most research to date centred on factors influencing the virulence, host range or impact on host populations of CDV (Haas et al.

1996; Roelke-Parker et al. 1996; Carpenter et al. 1998; Guiserix et al. 2007; Munson et al. 2008). Investigations on rabies virus revealed that two strains of the canid serotype 1 occur in the Serengeti that differ substantially in their virulence: a novel, apparently benign hyena strain was detected and a genetically distinct virulent strain was found in canids and viverrids (East et al. 2001). Lembo et al. (2007) reported cases of spotted hyenas infected with the virulent canid strain on the rim of the Crater and in rural areas surrounding the Serengeti National Park where spotted hyenas are sympatric with domestic dogs. They failed to detect any spotted hyenas infected with either the virulent or benign strain of rabies within the Serengeti National Park or on the floor of the Ngorongoro Crater, possibly because many of the brain samples they tested for rabies were screened using conventional methods (mouse inoculation and murine cells) and not by RT-PCR methods. Furthermore, a novel CoV variant was detected in hyenas that appeared to be of low virulence in hyenas and seemed to infect mainly young hyenas (East et al. 2004). Colleagues and I described a tick borne blood parasite *Hepatozoon* sp. that appeared to be benign in spotted hyenas but caused mortality in young hyenas below two months of age (East et al. 2008). *Streptococcus equi* subsp. *ruminatorum*, a bacterium closely related to a strain circulating in sheep and goats in Spain, was identified as responsible for clinical signs and even death in spotted hyenas inhabiting the Ngorongoro Crater (Höner et al. 2006; Speck et al. 2008).

In this study I established and applied several reverse-transcriptase polymerase chain reactions (RT-PCRs) for the detection of coronaviral (Chapter 3.1) and caliciviral (Chapter 3.2) RNA to screen a large set of faecal samples from individually known spotted hyenas and sympatric carnivores from the two study populations from different years. I used sequencing methods to characterise viral variants and determine possible evolutionary virus-host adaptations by testing for positive selection, determining non-conservative amino acid (aa) substitutions and generating hydrophobicity profiles.

I used statistical multinomial or binomial logistic regression models to investigate whether life history parameters as well as social and ecological factors such as age, sex, litter size, dominance status of littermates, social rank, concurrent infection with helminths or prey availability influenced infection status of individual hyenas. I used survival analysis to determine whether longevity of individuals differed between individuals only subjected to single infections with coronavirus or calicivirus or co-infections with both viruses. I documented patterns of simultaneous exposure to both viruses and the effects of infection on host individuals and variation in individual susceptibility (Chapter 3.3).

I investigated two hyena subpopulations inhabiting the Serengeti National Park and the adjacent Ngorongoro Crater to determine differences of pathogen prevalence and calculated the basic reproductive rate R_0 of infection with coronavirus to investigate viral persistence and extinction patterns in the two subpopulations (Chapter 3.4).

For the detection and characterisation of a tick-borne hemogregarine blood parasite *Hepatozoon* sp., I isolated DNA from an extensive set of blood and tissue samples from spotted hyenas as well as other carnivore species inhabiting the Serengeti ecosystem (Chapter 3.5). Additionally I screened blood and tissue samples obtained from African wild dogs *Lycaon pictus* for the presence of parvoviral and canine distemper viral nucleic acids (Chapter 3.6).

2 GENERAL MATERIAL AND METHODS

In this section I describe the standard methods I used in this study. Further details required by specific methods used in individual result chapters are described therein.

Study sites

The Serengeti ecosystem is defined as the area covered by the migratory movements of herbivores (Sinclair & Arcese 1995) and includes the Serengeti National Park as well as the adjacent Ngorongoro Crater. I included data in my study that were obtained from a metapopulation of free-ranging spotted hyenas including two subpopulations: one subpopulation inhabited the Serengeti National Park and the other inhabited the Ngorongoro Crater in northwestern Tanzania (Fig. 1). Climatically, the ecosystem falls within the seasonal tropics just south of the equator. Mean maximum temperatures are between 24° to 27°C, and mean minimum temperatures between 15° to 21°C. Mean annual rainfall in the Serengeti varies from 1050 mm in the northwest to 550 mm in the southeast (Sinclair et al. 2000). This rainfall is strongly seasonal, with peaks between March and May (long rainy season) and between November and December (short rainy season, Schaller 1972; Sinclair 1979). Rainfall is the main determinant of vegetation growth and hence ungulate food supply (Sinclair 1977; McNaughton 1979). The Ngorongoro Crater is a large (250 km²) caldera situated in the Ngorongoro Conservation Area. Both, the Serengeti (18000 km²) and the adjacent Ngorongoro Conservation Area are part of the Tanzanian sector of the Serengeti ecosystem. Although essentially self-contained, the Crater is physically linked to and accessible from the Serengeti plains and almost all herbivore and carnivore species that inhabit the Serengeti also occur in the Crater (Runyoro et al. 1995).

Study populations

The metapopulation of spotted hyenas used in this study was composed of the subpopulation in the Serengeti National Park that contained approximately 5300 individuals (Hofer & East 1995), and the subpopulation that inhabited the floor of the Ngorongoro Crater with approximately 400 individuals (Höner et al. 2005). Spotted hyenas in both the Serengeti and Crater subpopulations live in social groups called clans and share a similar social structure in that clans were stable, multi-female, multi-male, fission-fusion groups that contained natal adult females and their offspring, immigrant adult males and rarely ‘non-dispersing’, reproductively active adult natal males (Hofer & East 1993a; East & Hofer 2001; Höner et al. 2005, 2007).

Clans in both subpopulations defended group territories against neighbouring clans (Hofer & East 1993a; Höner et al. 2005).

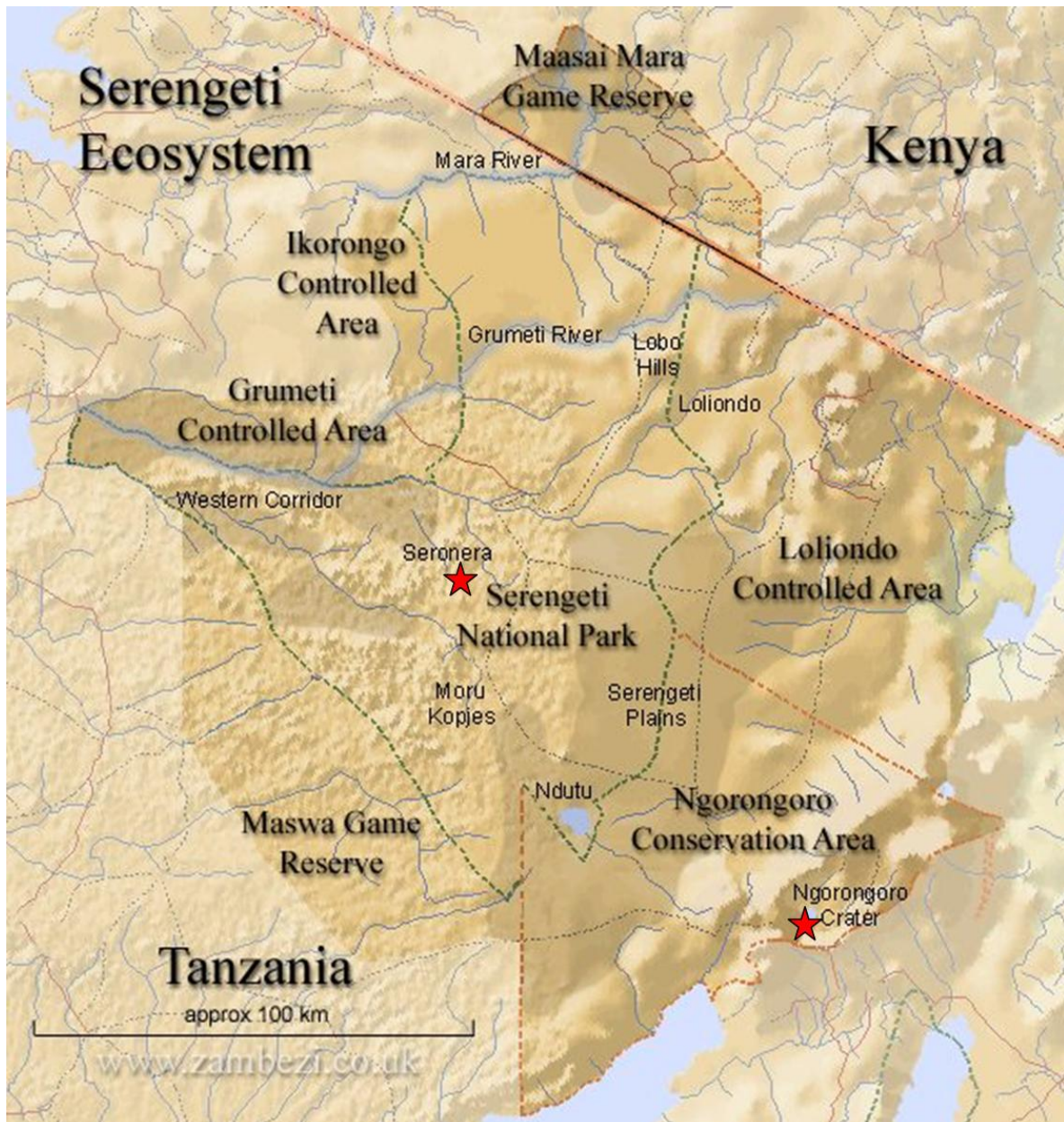


Figure 1: Map of the Serengeti ecosystem. Study sites (indicated by red stars) were situated in the centre of the Serengeti National Park and the Ngorongoro Crater in the Ngorongoro Conservation Area. Map modified after www.zambezi.co.uk.

Data included in my research were obtained from three spotted hyena clans in the Serengeti National Park that were closely monitored for approximately 22 years (May 1987- March 2009.), 20 years (November 1989 – March 2009) and 19 years (August 1990 – March 2009) and all (eight) clans resident on the Crater floor for approximately 12 years (April 1996/ February 1997 – December 2008). Members of all study clans were individually known (Hofer & East 1993a,b; Höner et al. 2005). Individual adult hyenas were recognised by their unique

spot patterns and other natural features such as scars and ear notches (Frank 1986; East et al. 1991; Hofer & East 1993a; Wachter et al. 2002). Young cubs were identified by scars, bald patches and ear notches. The sex of cubs was determined by the shape of the phallic glands (Frank et al. 1990). The age of cubs was determined to an accuracy of 7 days on the basis of pelage, size, locomotory abilities as well as shape and size of the ears (Pournelle 1965; Kruuk 1972; East et al. 1989). Adult female age was determined from estimated birth dates. I classified hyenas less than 365 days (< 12 months of age) of age as cubs, those between 365 and 729 days (12 to 24 months of age) as subadults and those 730 days of age or older (> 24 months of age) as adults. When cubs and subadults were pooled in one age group they were termed juveniles.

In both subpopulations there were separate linear dominance hierarchies among adult females and breeding, mostly immigrant males (East & Hofer 2001; Höner et al. 2007). Social status of individuals and the dominance status of cubs within litters were determined on the basis of an interaction matrix using aggressive actions such as lunging, chasing, biting, pushing, and submissive responses such as retreat, cower, ears down, tail between legs, in dyadic interactions recorded ad libitum at the den (East & Hofer 1991, 2001). On this basis, hyenas were assigned standardised ranks that were evenly distributed between the highest rank (+1) and the lowest rank (-1), with the median rank being scored as 0 (see East & Hofer 1991; Hofer & East 2008). Cubs typically establish a hierarchy in the first few days and weeks after birth by aggressive interactions resulting in a dominant and a subordinate cub (Smale et al. 1995; Drea et al. 1996; Hofer & East 1997).

All females breed, producing litters of one, two, or very rarely three cubs throughout the year (Wachter et al. 2002; Hofer & East 2008). Females usually only nurse their own offspring and lactation lasts for at least twelve months, during which time the offspring are kept at the communal den or dens inside clan territories where cubs rested together in narrow underground tunnels for many hours each day (Hofer & East 1995; Höner et al. 2005). Because adults cannot enter the narrow tunnels of spotted hyena communal dens (Kruuk 1972), cubs need to emerge from underground to be nursed by their mother and thus the presence and health status of even very young cubs can be determined during nursing bouts (East et al. 1989; Golla et al. 1999). Cubs stationed at the communal den are called den-based and do not venture far from the den, thus their home range is very restricted compared to older animals (Hofer & East 1993c; Höner et al. 2005). The communal den is the social centre of a clan where individuals, social interactions and the presence of clan members can be reliably observed (East & Hofer 1991; Hofer & East 1993c). Data on behaviour, demography and cubs

were recorded at the dens of all study clans at dusk (approximately between 1600 – 2000 hours) and dawn (approximately between 0530 – 1000 hours).

The main prey of both Serengeti and Crater hyenas are wildebeest *Connochaetes taurinus*, zebra *Equus burchelli*, Thomson's gazelle *Gazella thomsoni* and Grant's gazelle *Gazella granti* (Kruuk 1972; Holekamp & Smale 1995; Hofer & East 1997; Höner et al. 2005). In the Serengeti, the abundance of prey within group territories substantially fluctuates throughout the year owing to the movement of migratory herbivores. Migratory herds are usually absent from the territories for three-quarters of the year (Hofer & East 1993a), and during this time the density of resident herbivores inside the territory is low and cannot sustain all clan members (Hofer & East 1993a). Thus all members of Serengeti clans regularly left their territory individually or in small groups on short-term, long-distance foraging trips (up to 70km straight line distance from their territory) to concentrations of migratory herbivores, when migratory prey were absent from their territory (termed 'commuting trips', Hofer & East 1993a,b,c). Prey abundance in the Serengeti National Park was classified in three categories (see East & Hofer 1993a): (1) low prey abundance: only resident prey species, very low numbers; (2) medium prey abundance: resident prey and gazelles; (3) high abundance: resident prey and major migratory herds of wildebeest and zebra. Because the density of resident herbivores in the Ngorongoro Crater is more than one order of magnitude higher than in the Serengeti National Park (Hofer & East 1993a; Runyoro et al. 1995) Crater hyenas usually feed on the herbivores inside their own clan territory throughout the year, or in neighbouring territories of other Crater clans if they contain significantly higher densities of herbivores than present in their own territory (Höner et al. 2005).

Sample collection and isolation of pathogen nucleic acids

I benefitted from an extensive database containing life history and ecological data as well as a large set of samples of faeces, blood and tissues from my study population that were collected from individuals of three clans inhabiting the Serengeti National Park during the past 22 years as well as data collected from individuals of all eight clans inhabiting the Ngorongoro Crater during the past 12 years. Additionally I investigated samples that were obtained from 11 sympatric carnivore species belonging to the families Canidae, Felidae, Viverridae and Herpestidae (Table 1). These carnivores were living in close proximity to the hyena study clans in the Serengeti National Park and the Ngorongoro Crater or close to the Serengeti National Park border.

As my study was embedded in a long-term study on behaviour, it was important to establish non-invasive methods that can be conducted without affecting individual behaviour in the field and facilitated re-sampling. Methods necessary for the collection of blood or other tissue samples to determine prevalence of infection and pathogen exposure require interventions such as darting and anaesthesia and thus may have a variety of unwanted side effects, some of which might have a negative impact on the health status of handled wildlife (Hofer & East 1998). Blood and tissue samples were only obtained either from animals that died from natural causes or from animals that were immobilised in order to remove wire snares from poaching accidents (details on blood and tissue samples from hyenas and sympatric carnivores are contained in Appendix A).

Table 1: Number of samples obtained from hyena study clans and sympatric carnivores in the Serengeti ecosystem. Serengeti National Park (SNP), Ngorongoro Crater (NC).

Family	Species	Faecal samples	Blood & Tissue samples	Serum samples	Total
		N (SNP/NC)	N (SNP/NC)	N (SNP/NC)	N
Hyaenidae	spotted hyena (<i>Crocuta crocuta</i>)	350/86	85/19	87/9	636
Canidae	bat-eared fox (<i>Otocyon megalotis</i>)	9/0	13/0	-	22
	silver-backed jackal (<i>Canis mesomelas</i>)	17/0	2/1	-	20
	side-striped jackal (<i>Canis adustus</i>)	-	1/0	-	1
	African wild dog (<i>Lycaon pictus</i>)*	-	6/0*	-	6*
Felidae	serval cat (<i>Felis serval</i>)	-	2/0	-	2
	lion (<i>Panthera leo</i>)	-	11/1	-	12
Viverridae	common genet (<i>Genetta genetta</i>)	-	4/0	-	4
	civet (<i>Civettictis civetta</i>)	-	1/0	-	1
Herpestidae	white-tailed mongoose (<i>Ichneumia albicauda</i>)	-	3/0	-	3
	slender mongoose (<i>Galerella sanguinea</i>)	-	2/0	-	2
	dwarf mongoose (<i>Helogale parvula</i>)	-	1/0	-	1
Total		376/86	131/21	87/9	710

* samples obtained in the Loliondo Game Controlled Area close to the Serengeti National Park border

Faecal samples were collected immediately after defecation and were either stored and transported frozen at -80°C or preserved in RNAlater (Sigma-Aldrich Inc., St. Louis, MO, USA), initially stored at -10°C for up to four months, transported frozen and then stored at -80°C. Blood and tissue samples were collected from anaesthetised animals or animals found dead in the field and stored and transported as described for faecal samples. I used samples collected by colleagues during the long-term studies, and I collected additional samples from the study clans and sympatric carnivores in the Serengeti National Park during a field period between May and November 2007.

To homogenise faecal samples I used sterile glass balls and diluted the samples as a 10% solution in sterile DNase and RNase free DEPC treated water (Roth, Karlsruhe, Germany) and pulse vortexed each sample 10-15 seconds. I homogenised blood and tissue samples as a 10% solution in DEPC treated water or in buffer provided by DNA or RNA isolation kits (see below) using Precellys® 24 Homogenizer and ceramic beads (Peqlab Biotechnologie GmbH, Erlangen, Germany) that permit the disruption of cells and tissues without degradation of the RNA or DNA. After homogenisation I centrifuged the samples for 5 minutes at 13,000 rpm. I isolated pathogenic RNA and DNA simultaneously using the MinElute virus spin kit (Qiagen, Hilden, Germany) using 200 µl from the supernatant of the homogenates following the user manual instructions. In principle, the Qiaamp MinElute virus spin kit simultaneously isolates pathogenic RNA and DNA with a spin-column procedure. Nucleic acids bind specifically to the QIAamp MinElute silica-gel membrane while contaminants pass through. PCR inhibitors such as divalent cations, nucleases and proteins are completely removed in wash steps, leaving pure nucleic acids to be eluted in water. After elution of the nucleic acids, I stored samples at -80°C or continued processing them immediately by running (RT-) PCRs. When I applied additional or different methods, I describe them in the respective sections in the result Chapters.

(RT-) PCRs, sequencing and phylogenetic analysis

Wildlife pathogens often differ in their genetic sequence from pathogens already known from domestic animals. The first task of my study was to establish (RT-) PCR methods to detect pathogen variants circulating in Serengeti wildlife. Most studies on pathogens infecting African free-ranging carnivores are based on serological investigations that only document exposure to infections. Only little information on the molecular characterisation of pathogens was available. I therefore needed to establish reliable methods to screen non-invasively collected samples such as faeces which may contain substances or organisms such as bacteria, fungi or toxins that may act as inhibitors to or disturb PCR amplification. I concentrated on pathogens that infect the gastro-intestinal tract (CoV and calicivirus (CV)) in order to screen faecal samples of alive individuals, and on pathogens detectable in blood and tissue samples (*Hepatozoon* sp., CDV and CPV) available from carcasses or immobilised individuals.

In general I performed RT-PCRs for the detection of RNA viruses (CoV, Chapter 3.1, CV, Chapter 3.2; CDV, Chapter 3.6) using SuperScript™ III One-Step RT-PCR System with Platinum® Taq DNA Polymerase (Invitrogen, Karlsruhe, Germany) following the user manual's instructions with a total reaction volume of 25µl. I performed PCRs for the detection of nucleic acids from a DNA virus (CPV, Chapter 3.6) and a blood parasite (*Hepatozoon* sp.,

Chapter 3.5) using AccuPrime™ PCR System (Invitrogen, Karlsruhe, Germany) according to manufacturer's instructions with a total reaction volume of 25µl. Detailed information on primer sequences, their locations in the pathogenic genome, and on annealing temperatures are given in the respective chapters of the result section (coronavirus in chapter 3.1; calicivirus in chapter 3.2; *Hepatozoon* sp. in chapter 3.5; canine distemper virus in chapter 3.6). To confirm positive results I ran each (RT-) PCR at least in duplicate.

I analysed PCR amplification products by gel electrophoresis and visualised them by GelRed staining (Biotium Inc, Hayward, California). I purified PCR products of the expected length using Qiagen PCR purification Kit (Qiagen, Hilden, Germany) and sequenced PCR amplification products bidirectional using Big Dye Terminator Cycle sequencing kit 1.1 (Applied Biosystems [ABI], Darmstadt, Germany) following the manufacturer's instructions. I visualised the sequences on an ABI model 3130xl Genetic Analyzer (ABI). I edited, aligned and translated the obtained sequences as well as determined percentages of nucleotide (nt) and amino acid (aa) similarities with BioEdit 7.0.9.0 (Hall 1999). In general I used the neighbour-joining (NJ) method with different substitution models (maximum composite likelihood or Tamura-Nei Parameter) for nucleotide (nt) alignments and the Jones-Taylor-Thornton (JTT) matrix for amino acid (aa) alignments as implemented in MEGA 4.0 (Tamura et al. 2007). All tree constructions were done using 1000 bootstrap replications and in all tree figures only bootstrap values > 50 are shown. Which substitution model I chose for the respective data set is detailed in the respective result chapters. When I used methods other than those outlined here I explain them in detail in the respective result sections.

Data analysis

In this study I had a large data set that included different types of data: nominal categorical data on an arbitrary scale (e.g. infected or not infected; female or male), discrete categorical data on an ordinal scale (e.g. prey levels: low, medium, high), as well as data on a continuous scale (e.g. age in days, social status as absolute or standardised rank).

For analysing categorical data I used log-likelihood ratio tests ("chi-square analysis"), a well known non-parametric analysis to look for associations between parameters (e.g. relationship between age and prevalence of infection, Chapter 3.1, 3.2, 3.4 and 3.5; differences of prevalence of infection between years, Chapter 3.2 and 3.4).

For analysing data sets that included both categorical and continuous parameters, I constructed, if appropriate, complex individual models to test the respective hypotheses. To test which parameters may have an effect on infection status, I built binomial logistic regression models

that can be applied when the dependent variable is categorical and has only two categories (e.g. positive – negative; Chapter 3.2) and the independent = predictor = effect parameters are both on a categorical and continuous scales. I used a multinomial logistic regression model if the dependent variable had more than two categories (e.g., not infected, single coronavirus infection, single calicivirus infection, co-infection with both pathogens, Chapter 3.3). To investigate whether infection status had an influence on the longevity of individuals whose infection status was assessed whilst they were juveniles, I used survival analysis in the form of a Cox proportional hazards model. Survival analysis is an advanced general statistical method that allows the incorporation of “censored” data, i.e. data for which in some cases only a minimum estimate is available (e.g. for longevity if individuals were still alive at the end of the study). I applied this method for all data sets that contained censored data (e.g. animals that were infected and still alive, Chapter 3.3; duration of periods when cubs were present that were not observed until the period ended, Chapter 3.4).

I calculated the basic reproductive rate R_0 of coronavirus, the ability of coronavirus to persist in a completely susceptible population, or more specifically, the number of susceptible host individuals infected by one infected host individual. I applied existing formulas for the calculation of R_0 (Anderson & May 1979a,b, 1991) and applied them to separate age categories, as infection patterns differed between age categories (Chapter 3.4).

All statistical tests were performed using Systat 12.0 (Systat Software Inc., Richmond, USA) and probabilities are for two-tailed tests.

3 RESULTS

3.1 Genetic diversity, temporal change and host-species specificity of coronavirus strains in wild African carnivores

3.1.1 INTRODUCTION

Interest in coronaviruses (CoVs) that infect wild carnivore species increased as a result of the severe acute respiratory syndrom (SARS) CoV epidemic in 2003 and the identification of a SARS-like virus in the palm civet *Paguma larvata* (Guan et al. 2003). Interest subsequently waned when the likely zoonotic source of SARS-CoV was identified as Asian bat species in the genus *Rhinolophus* (Gorbalenya et al. 2006). As a result, current knowledge of the molecular phylogeny and global diversity of CoVs in wild carnivores remains limited even though they are common hosts of CoVs. For example, a SARS-like virus is known to infect raccoon dogs in China (Guan et al. 2003), a novel CoV was detected in Asian leopard cats *Prionailurus bengalensis* and Chinese ferret badgers *Melogale moschata* in southern China (Dong et al. 2007), and a novel CoV with a relatively higher similarity (80-84%) to feline CoV type II (FCoV-II) than to (76-78%) canine CoV type II (CCoV-II) was reported in spotted hyenas in Tanzania (East et al. 2004). There is considerable serological evidence of exposure to CoVs in several carnivore families, including the Felidae (e.g. European wild cat *Felis silvestris* in Europe, Leutenegger et al. 1999; Daniels et al. 1999; cheetah *Acinonyx jubatus* in Namibia, Heeney et al. 1990; Kennedy et al. 2003; Thalwitzer et al. 2010; lion *Panthera leo* in East Africa, Hofman-Lehmann et al. 1996), Hyaenidae (spotted hyena in East Africa, East et al. 2004; Harrison et al. 2004), Canidae (wolf *Canis lupus* in the USA, Zarnke et al. 2001; raccoon dog *Nyctereutes procyonoides* in China, Guan et al. 2003), Mustelidae (Chinese ferret-badger in China, Guan et al. 2003) and Viverridae (civet cat in China, Guan et al. 2003).

CoVs belong to the family Coronaviridae, in the order of Nidovirales, and are large, enveloped, single-stranded RNA viruses that cause enteric and respiratory diseases and central nervous system infections in avian and mammalian host species (Gorbalenya et al. 2006; Weiss & Navas-Martin 2005). CoVs have the largest genomes (27 to 31 kb) of all RNA viruses and during replication, recombination events frequently occur that typically involve closely related viruses (Herrewegh et al. 1998; Gorbalenya et al. 2006). The CoV genome contains large open reading frames (ORF1a, ORF1b) for non-structural proteins at the 5' end and four structural proteins and minor non-structural proteins at the 3' end of the genome. The

structural proteins include the spike (S) protein, the matrix (M) protein, the small envelope (E) protein and the nucleocapsid (N) protein (Weiss & Navas-Martin 2005; Buonavoglia et al. 2006). In this Chapter I focused on the relatively conserved M protein and more variable S protein. The M protein (about 29 kD) is the most abundant protein in the viral envelope and is required for virus assembly and the incorporation of the S protein into the viral envelope (de Haan et al. 1999; Escors et al. 2001). The S protein (150 – 200 kD) is responsible for the attachment of the virus to host cell receptors, and for fusion of both the viral envelope with the host cell membrane and cell-to-cell fusion. The structure of the S protein is thought to influence host species specificity (Lai & Holmes 2001; Kuo et al. 2000). The S protein also induces production of virus neutralising antibodies by the host and is thus likely to be important in virus-host evolution (Kuo et al. 2000; Gallagher & Buchmeier 2001; Decaro & Buonavoglia 2008) and thus subject to positive selection.

The Coronaviridae are divided into three main antigenic groups. This Chapter reports CoVs in African wild carnivores that belong to Group 1 which includes FCoV, CCoV, the porcine CoVs transmissible gastroenteritis virus (TGEV) and porcine epidemic diarrhoea virus (PEDV), as well as human CoV (HCoV 229E) (e.g. Weiss & Navas-Martin 2005; Decaro & Buonavoglia 2008) and ferret CoV (Wise et al. 2006). CCoV strains are differentiated into genotypes I (CCoV-I) and II (CCoV-II) based on differences in the genes encoding for the S protein, M protein and the ORFs (Decaro & Buonavoglia 2008). The S gene of CCoV-I has a high similarity with FCoV type I (FCoV-I) whereas the S gene of CCoV-II has a higher similarity to TGEV of swine (Pratelli et al. 2004; Decaro et al. 2009). Although both CCoV-I and CCoV-II generally cause relatively mild enteric infections in domestic dogs with more severe clinical signs in young animals (Pratelli 2006), some CCoV-I and II strains may also cause virulent infections in domestic dogs (Pratelli et al. 2002; Sanchez-Morgado et al. 2004; Renhofer et al. 2005; Buonavoglia et al. 2006).

FCoV strains are also divided into genotypes I and II, and these feline enteric CoVs typically cause unapparent to mild enteritis (Herrewegh et al. 1998). More virulent FCoV strains may cause feline infectious peritonitis (FIP), a typically fatal disease in domestic cats (Vennema et al. 1998). FCoV-II is thought to have arisen from double homologous RNA recombination events between FCoV-I and CCoV (Herrewegh et al. 1998) and is perhaps the most often quoted example of the evolution of a new CoV strains through homologous recombination. The S protein of FCoV-II strains has a higher similarity to CCoV and TGEV strains than to FCoV-I strains (Wesseling et al. 1994).

The aim of this chapter was to determine the genetic diversity, phylogenetic relationships and host species specificity of CoV strains circulating in spotted hyenas and sympatric wild carnivore species within a natural ecosystem. For this I investigated fresh faecal samples that were obtained in a non-invasive manner, from carnivore species belonging to the families Hyaenidae and Canidae that inhabited a central area in the SNP and the floor of the NC. I screened faecal samples for the presence of coronaviral RNA and sequenced fragments of coronaviral M and S genes from positive samples. To determine viral diversity and the phylogenetic relationship of these coronaviral sequences I used nucleotide (nt) and amino acid (aa) sequences from a 369 nt long (deduced 122 aa) fragment of the M gene. To investigate host-virus adaptations I considered synonymous and non-synonymous nt substitutions as well as conservative and non-conservative aa substitutions that might indicate positive selection, and hydrophobicity profiles that predict viral surface antigenic regions to document possible host specificity (Hopp & Woods 1983) using a 618 nt long (deduced 206 aa) fragment of the S gene. Based on a previous study (East et al. 2004) I expected spotted hyenas to be infected with FCoV-II-like strains, whereas I expected the two other potential host species that belong to the family Canidae (silver-backed jackals *Canis mesomelas* and bat-eared foxes *Otocyon megalotis*), to be infected with CCoV-like strains. I show that hyenas can be infected by both FCoV-II-like, TGEV-like and CCoV-II-like strains and thus harboured a considerable diversity of CoV strains within a short period of a few years. I provide evidence of positive selection at aa sites in the partial S protein and for CoV host-species specificity. I detected an unexpected diversity and rapid temporal change of CoV group 1 variants within a wild carnivore species in the SNP which has, to my knowledge, not previously been described in any other wild carnivore species.

3.1.2 METHODS

Samples

Between 1995 and 2007, 350 faecal samples were collected immediately after deposition from 218 individually known spotted hyenas that were members of three closely monitored large clans in the Serengeti National Park (East et al. 2003; East et al. 2008). Samples were stored as described in Chapter 2. Each clan defended a territory of approximately 55 km² that contained a communal den (Hofer & East 1993a). I considered two age categories: hyenas younger than 12 months of age termed cubs (n = 123), and hyenas older than 12 months of age (n = 95). Hyena cubs were chiefly confined to the immediate vicinity of the communal den, whereas older animals ranged far more widely both within and beyond the clan terri-

tory (Hofer & East 1993c). Additionally I screened 86 faecal samples from 82 individually known spotted hyenas from all eight clans inhabiting the adjacent Ngorongoro Crater (42 cubs and 40 older individuals) collected between 1997 and 2005. Furthermore I screened 85 blood and tissue samples from Serengeti hyenas (tissues: $n = 47$; blood $n = 38$) and 19 tissue samples from Crater hyenas (details on sorts of tissues are contained in Appendix A). The birth date for all animals born in the study clans was estimated using several age-related changes in appearance and proficiency in movement following the methods detailed in East et al. (2003) and Chapter 2. During this study, all communal dens were occupied by at least a few, and much more frequently by many cubs.

Additionally, between 2003 and 2007, 17 faecal samples were collected from adult silver-backed jackals and between 2004 and 2008, nine faecal samples were obtained from adult bat-eared foxes *Otocyon megalotis* that inhabited the territories defended by the spotted hyena clans in the Serengeti monitored during this study.

RT-PCR and sequencing of PCR products

For the amplification of the target sequences of CCoV-I and II as well as of FCoV-II strains I used previously published primer pairs to amplify a 409 nt long fragment from the M gene of CCoV-I and II using primers CCoV1 and CCoV2 (Pratelli et al. 2002; Benetka et al. 2006) and a 694 nt long fragment of the S gene of CCoV-II and FCoV-II using primers S5 and S6 (Pratelli et al. 2004). RT-PCR amplification was successful in hyena samples collected in the Serengeti from 26 cubs and 5 individuals older than 12 months of age. Thirteen samples obtained from hyenas (including two spotted hyena cubs sampled in 2004, one spotted hyena cub sampled in 2006, nine spotted hyena cubs and one adult sampled in 2007, plus one sample from a silver-backed jackal in 2007) contained sufficient RNA for further sequencing procedure. All other positive samples were considered as positive as they showed PCR amplicons of the expected length after gel electrophoresis with both primer pairs in duplicate RT-PCRs.

Phylogenetic analysis

For editing and translating the sequences as well as the determination of the percentages of nt and aa similarities I used BioEdit 7.0.9.0 (Hall 1999). M gene sequences of the expected length (369 nt) from all PCR products and S gene sequences of the length between 618 nt and 648 nt are contained in Appendix B. I compared the sequences obtained in this study to published sequences from M and S gene fragments from CoVs of feline, canine and porcine hosts entered in GenBank. GenBank accession numbers of published sequences are as follows: canine CoVs: CCoV 430/07 (EU924790), CCoV 341/05 (EU856361), CCoV CB/05

(DQ112226), CCoV 119/08 (EU924791), CCoV 174/06 (EU856362); feline CoVs: FECV 79-1683 (Y13921), FIPV 79-1146 (DQ010921) and porcine CoVs: TGEV virulent Purdue (DQ811789), TGEV PUR46-MAD (AJ271965) and TGEV Miller M60 (DQ811786).

To assess phylogenetic relationships, I used the neighbour-joining (NJ) method based on a maximum composite likelihood parameter and maximum parsimony (MP) for nt alignments, and a Jones-Taylor-Thornton matrix for aa alignments as implemented in MEGA 4.0 (Tamura et al. 2007). In addition I used maximum-likelihood (ML) method as implemented in PAUP (Swofford 2002; ML analysis using PAUP was done by Dr. Jörn Fickel, Leibniz Institute of Zoo and Wildlife Research (IZW), Berlin, Germany) to confirm the robustness of the nt trees. The DNA sequence alignment of the M gene was based on a 369 nt fragment, the S gene sequence alignment was based on a 618 nt fragment.

Analysis of virus-host adaptations and evolutionary selection in the S gene fragments

Sections of nucleic acids that evolve slowly exhibit relatively conserved nt sequences in related organisms. Mutations in these sections are likely to affect functions subject to negative selection so that organisms with mutations in these sections may suffer functional impairment and poor survival. Sections of the genome that are evolving rapidly are unlikely to be conserved among related organisms and are likely to affect functions subject to some degree of positive selection. Organisms with mutations in these sections of the genome are functionally improved and thus have a selective advantage. In molecular evolutionary studies, nt substitutions can be partitioned into two classes: synonymous substitutions that cause no change in the aa sequence, and non-synonymous substitutions that result in an aa replacement. To test for positive selection the number of non-synonymous substitutions (dN) and synonymous substitutions (dS) are determined and a ratio of $dN/dS < 1$ indicates negative selection whereas a ratio of $dN/dS > 1$ indicates positive selection. I calculated in the antigenically relevant S gene fragments obtained from spotted hyenas site-specific dN/dS scores using the software package JCoDa v. 1.0. (Steinway et al. 2010).

Conservative aa substitutions are defined as the replacement of one aa by another aa with similar biochemical and physical characteristics to the original aa. Such substitutions are unlikely to influence the structure of the protein formed by this section of the genome. In contrast, non-conservative aa substitutions, in which one aa is replaced by another aa that has markedly different biochemical and physical characteristics, are likely to cause changes in the protein structure. Such changes in the coronaviral S protein may potentially influence the mechanism of virus-host cell attachment and thus lead to changes in host specificity. I deter-

mined conservative and non-conservative aa substitutions using the conserved property difference locator (CPDL) key (Mayer et al. 2005).

Additionally, I used aa sequence data from S gene fragments to predict hydrophobicity profiles based on the assumption that hydrophilic regions are predominantly surface-orientated and therefore potentially antigenic (Hopp & Woods 1983; Welling et al. 1985). I used algorithms developed by Hopp & Woods (1983) as implemented in CLC Protein Workbench 5.1 (CLC Bio, Aarhus, Denmark). Since the approximate size of an antigenic determinant is 6-7 aas, I have chosen a window size of 7 aas (Welling et al. 1985) across the investigated S gene fragment. In the generated profiles, peaks above 0 (see Fig. 6) are considered to be hydrophilic and thus are regarded as potential antigenic regions (Hopp & Woods 1983). Comparison of hydrophobicity profiles of different viral variants provides an insight into possible antigenic differences between strains which in turn may influence viral-host dynamics and host specificity.

Statistical analysis

I performed a log-likelihood ratio test to examine differences in the shedding prevalence of coronaviral RNA in the faeces among cubs (< 12 months) and older animals (\geq 12 months).

3.1.3 RESULTS

The effect of age on the prevalence of infection

In the samples obtained from hyenas inhabiting the Serengeti National Park I detected coronaviral RNA significantly more often in faeces from cubs (26 positive faeces from 123 cubs, 21.1%) than in faeces from animals older than 12 months (five positive faeces from 95 individuals older than 12 months of age, 5.3 %; log-likelihood ratio $\chi^2 = 12.24$, d.f. = 1, $n = 218$, $p = 0.0005$). None of the samples obtained from hyenas from the Ngorongoro Crater were positive. The 31 Serengeti spotted hyenas with coronaviral RNA in their faeces were members of all three clans studied. Ten identical spotted hyena CoV sequences obtained in 2007 came from members of all three clans and thus infection with this viral type was widespread and not restricted to a single social group. I also detected coronaviral RNA in one of 17 faecal samples (5.9% positive) from adult silver-backed jackals, indicating a similar prevalence of viral excretion in adult jackals and spotted hyenas older than 12 months of age. Of nine faecal samples from adult bat-eared foxes that I screened none were positive.

Phylogeny, host specificity and diversity of M gene fragments

I obtained 14 sequenced CoV M gene fragments from hyena hosts between 2004 and 2007 and one M gene fragment from a jackal host in 2007. To assess the phylogenetic relationship between CoVs infecting carnivore hosts in the Serengeti National Park between 2004 and 2007 I compared sequences of the M gene with a length of 369 nt. One fragment from 2006 and nine of ten fragments from 2007 had identical sequences and are thus collectively referred to as ‘CoV Hyenas 06/07’ (Table 2). These identical sequences were recovered from faeces collected in all three study clans inhabiting the Serengeti National Park. This group of identical sequences differed from three fragments obtained from hyenas in 2004 and also from one other fragment collected from a hyena in 2007 (CoV Hyena 91/07). The M gene fragment from a jackal in 2007 differed from all M gene fragments obtained from hyenas, including those obtained in 2007. One fragment from a hyena in 2004 (CoV Hyena 42/04-1) clustered with

Table 2: Comparison of CoV M gene nt sequences (369 nt) and deduced aa sequences (122 aa) obtained from faeces from spotted hyenas and a silver-backed jackal with published canine, feline and porcine CoVs.

CoVs	Hyena 36/04		Hyena 42/04 1		Hyena 42/04 2		Hyena 91/07		Hyenas 06/07 ¹		Jackal 07	
	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
Hyena/Jackal CoVs												
Hyena 36/04	100	100	80.7	86.0	97.0	98.3	97.8	97.5	98.0	98.3	98.3	99.1
Hyena 42/04-1			100	100	81.5	85.2	79.6	84.4	79.8	85.2	79.6	86.0
Hyena 42/04-2					100	100	95.3	97.5	95.6	98.3	95.9	99.1
Hyena 91/07							100	100	99.7	99.1	98.3	98.3
Hyenas 06/ 07 ¹									100	100	98.6	99.1
Jackal 07											100	100
Canine CoVs												
CCoV ²	98.3	99.1	79.6	86.0	95.9	99.1	98.3	98.3	98.6	99.1	100	100
CCoV 430/07	98.3	99.1	80.1	86.0	96.4	99.1	98.3	98.3	98.6	99.1	98.3	100
CCoV 119/06	98.0	99.1	79.8	86.0	95.6	99.1	98.0	98.3	98.3	99.1	99.7	100
CCoV 174/06	97.5	99.1	81.2	86.0	95.1	99.1	97.5	98.3	97.8	99.1	98.0	100
Feline CoVs												
FECV 79-1638	80.4	86.0	99.1	100	81.7	85.2	79.3	84.4	79.6	85.2	79.3	86.0
FIPV 79-1146	80.4	86.0	91.0	98.3	80.7	85.2	79.3	84.4	79.6	85.2	79.3	86.0
Porcine CoVs												
TGEV ³	94.2	97.5	79.6	84.4	92.3	96.7	94.2	95.9	94.2	96.7	95.1	97.5
TGEV Miller M60	94.0	98.3	78.8	85.2	92.3	97.5	94.2	96.7	94.2	97.5	95.1	98.3

¹: CoV sequences from hyenas in 2006 and 2007 were identical and grouped as ‘CoV Hyena 06/07’

²: Canine CoV sequences of CCoV CB/05 and CCoV 341/05 were identical and grouped as ‘CCoV’

³: TGEV virulent Purdue and TGEV PUR46-MAD were identical and grouped as ‘TGEV’

FCoV-II strains. The two other fragments from 2004 (CoV Hyena 42/04-2 and CoV Hyena 36/04) were positioned separately and basal to the TGEV cluster (Fig. 2). All 11 fragments collected from hyenas in 2006 and 2007 formed a distinct cluster most closely related to the CCoV-II cluster (Fig. 2). The one M gene fragment from a jackal host in 2007 clustered with

CCoV-II strains from domestic dogs from Europe (Fig. 2). Together, these results indicate a considerable diversity of CoV strains within one host species during four years.

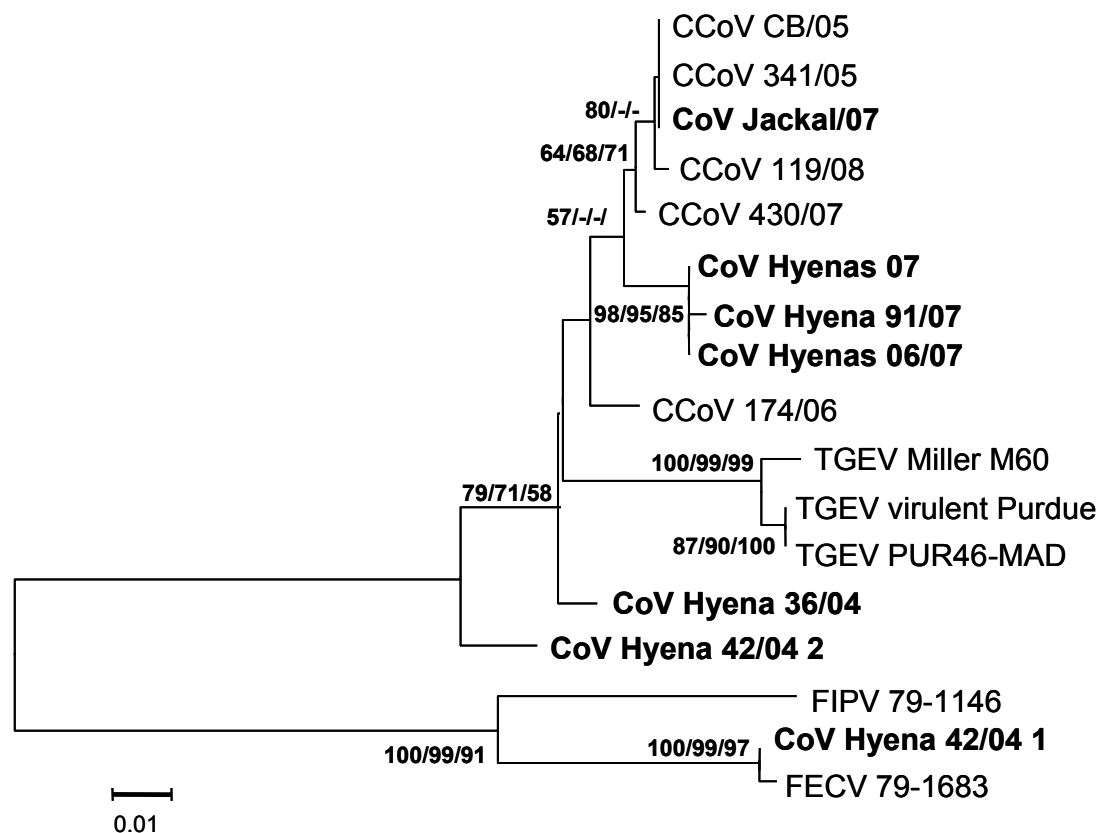


Figure 2: Phylogenetic relationship between CoV strains from spotted hyenas, a silver-backed jackal and published canine (CCoV), feline (FIPV/FECV) and porcine (TGEV) CoVs based on a 369 nt fragment of the M gene. Unrooted NJ tree constructed using maximum composite likelihood model. Numbers at nodes represent the percentage of 1000 bootstrap replicates. Bootstrap values are given for NJ/MP/ML and values < 50 are not shown. Scale bar indicates the estimated number of nt substitutions per site.

Comparison of the nt sequence of the M gene fragment CoV Hyena 42/04-2 with that of CoV Hyena 42/04-1 which were both retrieved from one hyena faecal sample, revealed 68 nt and 18 aa substitutions (Fig. 3) resulting in a low aa similarity of only 85.2 % (Table 2). This result provided evidence of simultaneous infection of one hyena with two CoV strains. The hyena faecal sample that contained two different M gene sequences yielded only one S gene sequence (CoV Hyena 42/04, see Table 3). Fragment CoV Hyena 42/04-1 had a high nt similarity (1 nt substitution, 99.1%) and identical aa sequence to the published FCoV-II strain FECV 79-1638 (Table 2), whereas the other M gene fragment CoV Hyena 42/04-2 from the same host individual had the highest nt sequence similarity to the M gene fragment CoV Hyena 36/04 collected from another hyena in 2004 (Table 2). Combined these results indicate that in 2004, hyenas were infected with an FCoV-II like strain and two other strains more similar to CCoV-II than FCoV-II (Table 2). All M gene fragments obtained from hyenas in 2004 were distinct from the eleven M gene fragments obtained from the same hyena clans in

2006 and 2007. In 2006 and 2007 I only obtained evidence of infection in hyenas with CCoV-II like strains (Table 2).

Non-conservative aa substitutions in the M protein

Comparison of M gene sequence data from a 122 aa fragment from CoV strains identified during this study and published information from strains retrieved from domestic canine, feline and porcine hosts revealed four unique non-conservative aa substitutions in strains obtained from hyenas (site 121: cysteine (C) instead of glycine (G); site 127: threonine (T) instead of isoleucine (I); site 204: glutamine (Q) instead of lysine (K); site 219: phenylalanine (F) instead of leucine (L); indicated by black triangles in Fig. 3). The fragment from the jackal contained no aa substitutions in comparison to the reference strain (Fig. 3).

	120	130	140	150	160	170	180	190
CCoV CB/05	FSVAGAI	VTFLWIMYFVRS	IQLYRRTKS	UWSFNPETN	AILCVS	ALGRSYVLP	LEGVPTGVT	LTLLSGNLYAEGFKIAGG
CoV Hyena 36/04	T.....
CoV Hyena 42/04 1	V...A..M.....	N.....	D.T.....M...
CoV Hyena 42/04 2
CoV Hyenas 06/07	C.....
CoV Hyena 91/07	C.....
CoV Jackal 07
CCoV 430/07
CCoV 341/05
CCoV 119/08
CCoV 174/06
FECV 79-1638	V...A..M.....	N.....	D.T.....M...
FIPV 79-1146	V...A..M.....	V.....	N.....	D.T.....M...
TGEV virulent Purdue	..I.....	V.....	K.....
TGEV PUR46-MAD	..I.....	V.....	K.....
TGEV Miller M60	..I.....	V.....

	200	210	220	230
CCoV CB/05	MNIDNLP	KYVMVALPS	RTIVYTLV	GKKLKASSATGWAYYVKS
CoV Hyena 36/04
CoV Hyena 42/04 1	LT.EH.....	I.T.N.....	Q...TT.....
CoV Hyena 42/04 2	F.....
CoV Hyenas 06/07
CoV Hyena 91/07	Q.....
CoV Jackal 07
CCoV 430/07
CCoV 341/05
CCoV 119/08
CCoV 174/06
FECV 79-1638	LT.EH.....	I.T.N.....	Q...TT.....
FIPV 79-1146	LT.EH.....	I.T.....	Q...TT.....
TGEV virulent Purdue
TGEV PUR46-MAD
TGEV Miller M60

Figure 3: Alignment of M protein fragment sequences of 122 aa in length from CoV strains obtained from spotted hyenas, a silver-backed jackal and published CCoVs, feline CoVs (FCEV and FIPV) and TGEVs. Sequences were aligned using CB/05 as the reference strain. ▼: non-conservative aa substitutions.

no aa substitutions in comparison to the reference strain (Fig. 3). The sequence CoV Hyena 42/04-1 had the same aa substitutions as the FCoV strain FECV 79/-1638 (Fig. 3).

Coronavirus phylogeny based on S gene fragments

In a phylogenetic analysis based on nt sequences of the S gene fragment, both fragments from hyenas in 2004 clustered more closely with FCoV-II and TGEV, whereas fragments collected from hyenas in 2006 and 2007 plus the fragment obtained from a jackal in 2007 clustered with CCoV-II strains (Fig. 4a). However, in an analysis based on the deduced aa sequences of the S gene fragments, both fragments collected from hyenas in 2004 clustered together and were placed closer to fragments collected from hyenas in 2007, than to a fragment from a hyena in 2006 and from a jackal in 2007 (Fig. 4b).

All aa sequences of S gene fragments obtained from hyenas were more similar to most published CCoV-II strains than to published FCoVs and TGEVs (Table 3). The two S gene fragments in 2004 had a higher aa sequence similarity (99.0 %) to each other than to fragments obtained from hyenas in 2006 and 2007 (Table 3). The fragments from hyena faeces in 2006 and 2007 also had a high aa sequence similarity (97.0 %). The aa sequence of the S gene fragment collected from the jackal in 2007 had a higher similarity to published CCoV-IIs than to the fragment sequences from sympatric hyena hosts in the same year (Table 3).

Table 3: Comparison of CoV S gene nt sequences (618 nt) and deduced aa sequences (206 aa) obtained from faeces from spotted hyenas and a silver-backed jackal with published canine, feline and porcine CoVs.

	Hyena 36/04		Hyena 42/04		Hyena 64/06		Hyenas 07 ¹		Jackal 07	
CoVs	nt	aa	nt	aa	nt	aa	nt	aa	nt	aa
Hyena/Jackal CoVs										
CoV Hyena 36/04	100	100	99.6	99.0	91.5	97.0	91.7	96.6	93.0	97.0
CoV Hyena 42/04			100	100	91.5	97.0	91.7	97.0	93.0	97.0
CoV Hyena 64/06					100	100	98.8	97.0	93.8	97.5
CoV Hyenas 07 ¹							100	100	93.6	96.6
CoV Jackal/07									100	100
canine CoVs										
CCoV CB/05	93.8	97.5	93.8	97.5	94.6	98.0	94.4	97.0	98.5	99.5
CCoV 341/05	92.7	97.5	92.7	97.5	97.2	98.5	96.7	97.5	95.9	98.0
CCoV 430/07	91.1	96.6	91.1	96.6	94.8	98.0	94.3	97.0	93.5	97.5
CCoV 119/08	93.5	97.5	93.5	97.5	94.3	98.0	94.1	97.0	99.1	99.5
CCoV 174/06	91.4	97.5	91.4	97.5	95.6	98.5	95.4	97.5	94.4	98.0
feline CoV²										
FIPV 79-1146	93.0	96.1	93.0	96.1	90.4	96.6	90.2	95.6	90.7	97.0
porcine CoVs										
TGEV virulent Purdue	92.2	95.1	92.2	95.1	91.7	96.6	91.2	95.6	92.7	97.0
TGEV Miller M60	91.9	94.6	91.9	94.6	91.1	96.1	90.9	95.1	92.0	96.6
TGEV PUR46-MAD	92.2	95.1	92.2	95.1	91.7	96.6	91.2	95.6	92.7	97.0

¹ Identical S gene sequences from hyena CoVs from 2007 were grouped as 'CoV Hyena 07'

² FECV 79-1638 was excluded from analysis (no published S gene sequence available in GenBank)

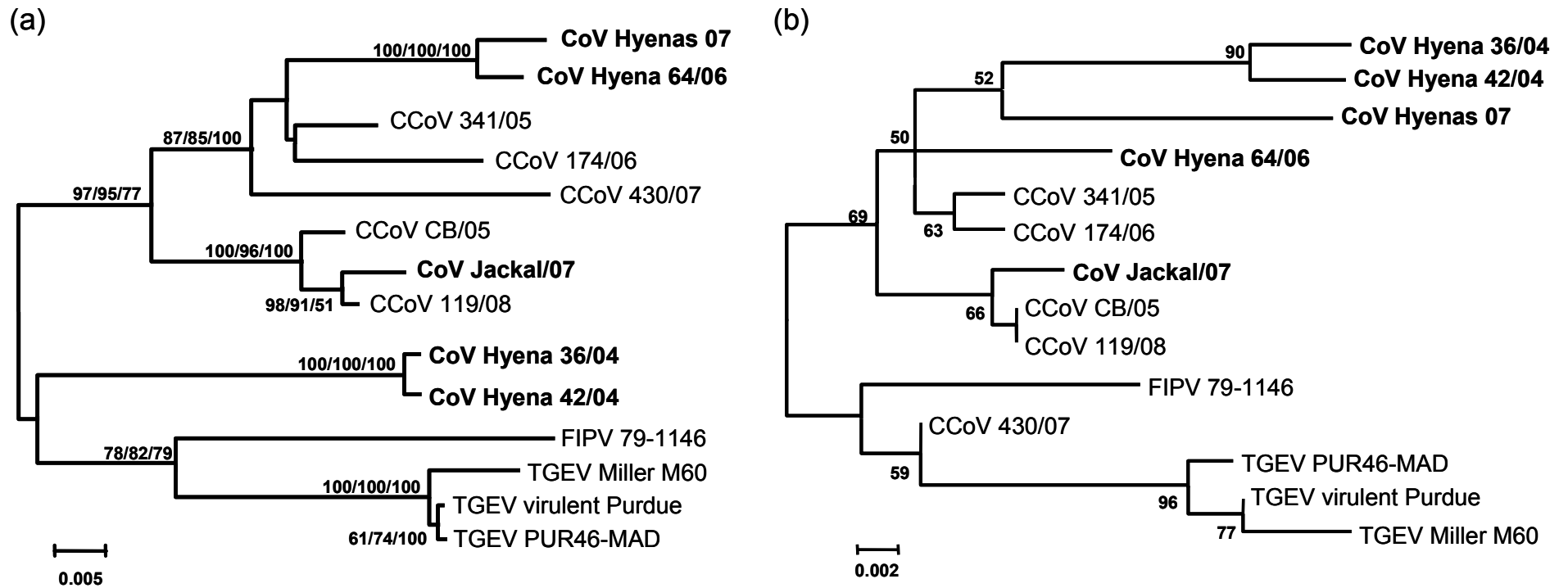


Figure 4: Phylogenetic relationship between CoV S gene fragments obtained from spotted hyenas, a silver-backed jackal and published canine, feline and porcine CoVs. (a) NJ tree based on 618 nt long fragments of the S gene. The tree was constructed using a maximum composite likelihood model. Numbers at nodes represent the percentage of 1000 bootstrap replicates. Bootstrap values are given for NJ/MP/ML. Scale bar indicates the estimated number of nt substitutions per site. (b) NJ tree based on deduced 206 aa of the S gene fragment. The tree was constructed using the JTT matrix. Numbers at nodes represent the percentage of 1000 bootstrap replicates. Scale bar indicates the estimated number of aa substitutions per site.

Non-conservative aa substitutions in the S gene fragment

Comparison of aa sequences of coronaviral S gene fragments from hyenas with published data on strains from domestic animal hosts revealed eight unique aa substitutions that were only present in sequences obtained from hyenas seven of which were non-conservative (at sites: 1179 and 1248: S instead of alanine (R); 1186: methionine (M) instead of K; 1247: A instead of aspartic acid (D); 1267: G instead of D; 1281: A instead of valine (V); 1289: R instead of Q; indicated as black triangles in Fig. 5). Interestingly CoV Jackal 07 had a non-conservative substitution which is also present in TGEV strains (at site 1337 F instead of L, Fig. 5).

Hydrophobicity profiles and site-specific positive selection in S gene fragments

Hydrophobicity profiles generated from S gene fragment sequences revealed likely changes in the hydrophobicity of potential antigenic regions associated with aa substitution sites (Fig. 6). Within the relatively short profiles examined, the profile of CoV Hyena 36/04 (Fig. 6a) was most dissimilar (mainly in an initial 0 – 20 aa section and central section 60 – 120 aa) to other S fragment profiles collected from hyena (CoV Hyena 42/04, Fig. 6b; CoV Hyena 06, Fig. 6c; CoV Hyenas 07, Fig. 6d), and the fragment profile obtained from the jackal (CoV Jackal/07, Fig. 6e). CoV Hyenas 07 (Fig. 6d) had a unique structure in section aa 105 – 120. The profile CoV Hyena 06 (Fig. 6c) was similar to CoV Jackal/07 (Fig. 6d).

Site-specific dN/dS scores detected nine possible aa sites in S gene fragments collected from hyenas that were under positive selection (sites 1247 and 1248 are covered by one peak; Fig. 7). Six of these sites were positions of non-conservative aa substitutions (at sites 1179, 1186, 1247, 1248, 1267, 1281 and 1289 in Fig. 5). Site 1247 and 1281 were also sites of conservative substitutions, as were sites 1326 and 1380 (Fig. 5). Differences in hydrophobicity profiles (Fig. 6a-d) corresponded to the sites that were under positive selection (Fig. 7).

	1180	1190	1200	1210	1220	1230
CCoV CB/05	VRASRQLAKDKVNECVRSQSQRFGCGNGTHLFSLANAAPNGMIFFHTVLLPTAYETVTA					
CoV Hyena 36/04M.....					
CoV Hyena 42/04					
CoV Hyena 64/06					
CoV Hyenas 07	.S.....					
CoV Jackal 07					
CCoV 430/07					
CCoV 341/05					
CCoV 119/08					
CCoV 174/06					
FIPV 79-1146					
TGEV virulent Purdue					
TGEV PUR46-MAD					
TGEV Miller M60					

	1240	1250	1260	1270	1280	1290
CCoV CB/05	WSGICASDGDRTFGLVVKDVQLTLFRNLDDKFYLTPTRTMYQPRVATSSDFVQIEGCDVLF					
CoV Hyena 36/04S.....G.....A.....					
CoV Hyena 42/04AS.....G.....A.....					
CoV Hyena 64/06				I.....	
CoV Hyenas 07NS.....					R.....
CoV Jackal 07					
CCoV 430/07					
CCoV 341/05E.....					
CCoV 119/08					
CCoV 174/06N.....					
FIPV 79-1146					
TGEV virulent Purdue	.A.....					
TGEV PUR46-MAD	.P.....					
TGEV Miller M60	.A...L.....					

	1300	1310	1320	1330	1340	1350
CCoV CB/05	VNATVIELPSIIPDYIDINQTVQDILENYRPNWTVPELTLDFNATYLNLTGEINDLEFR					
CoV Hyena 36/04D.....					
CoV Hyena 42/04D.....					
CoV Hyena 64/06D.....F.....					
CoV Hyenas 07D.....F.....					
CoV Jackal 07				F.....	
CCoV 430/07D.....F.....D.....					
CCoV 341/05D.....F.....					
CCoV 119/08					
CCoV 174/06D.....F.....					
FIPV 79-1146D.....E.....F.....D.....					
TGEV virulent PurdueSD.....F.....F.....D.....					
TGEV PUR46-MADSD.....F.....F.....D.....					
TGEV Miller M60SD.....F.....F.....D.....					

	1360	1370	1380
CCoV CB/05	SEKLHNTTVELAVLIDNINNTLVNLE		
CoV Hyena 36/04		
CoV Hyena 42/04		
CoV Hyena 64/06I.....		
CoV Hyenas 07		
CoV Jackal 07		
CCoV 430/07I.....		
CCoV 341/05		
CCoV 119/08		
CCoV 174/06		
FIPV 79-1146I.....		
TGEV virulent PurdueI.....		
TGEV PUR46-MADI.....		
TGEV Miller M60I.....		

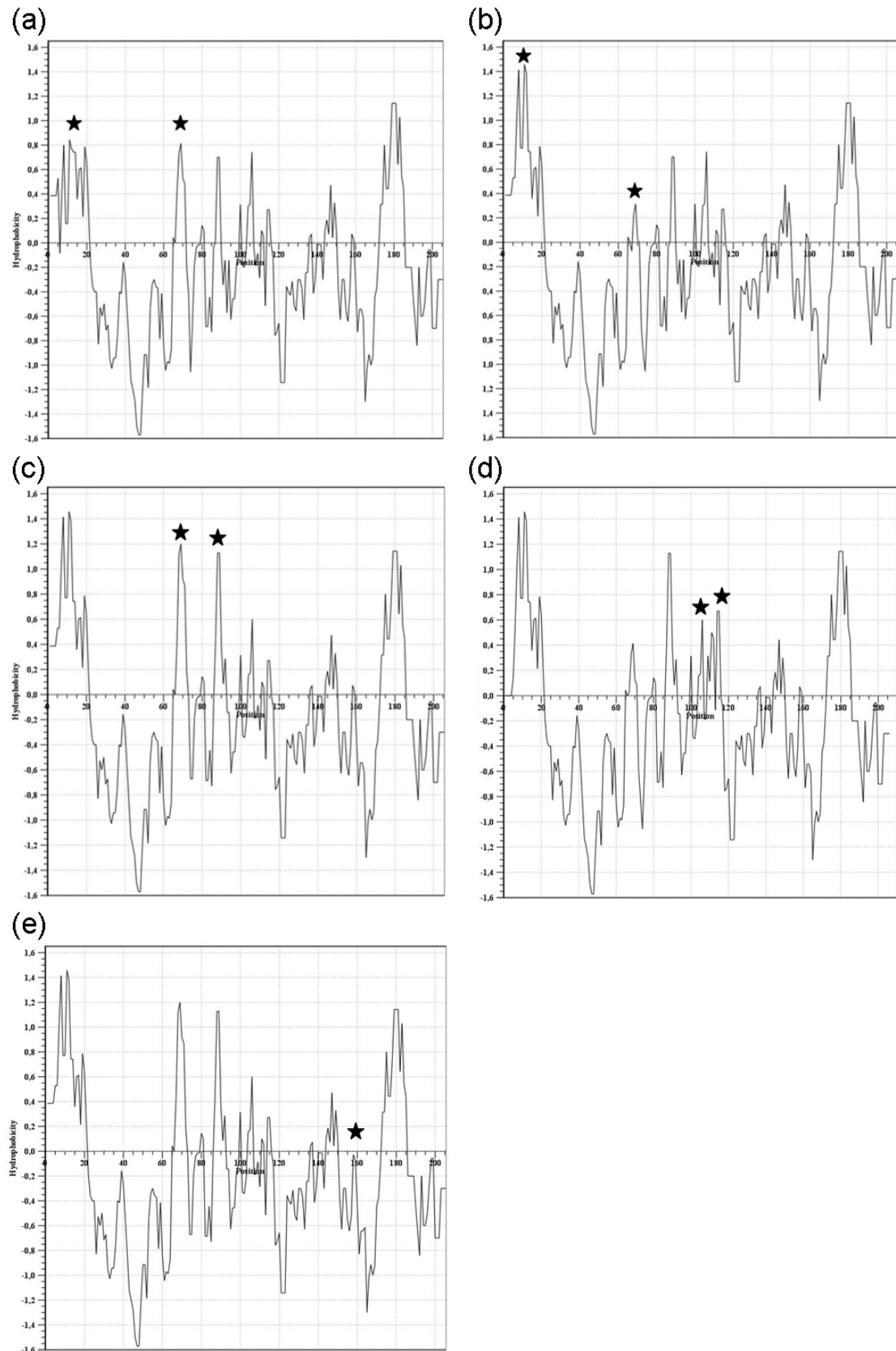


Figure 6: Hydrophobicity profiles based on sequence data from CoV S protein fragments (206 aa) obtained from Serengeti carnivores. (a) CoV Hyena 36/04, (b) CoV Hyena 42/04, (c) CoV Hyena 64/06, (d) CoV Hyenas 07 (e) CoV Jackal 07. Peaks above 0 suggest hydrophilic antigenic regions. Stars indicate peaks that differ between some strains.

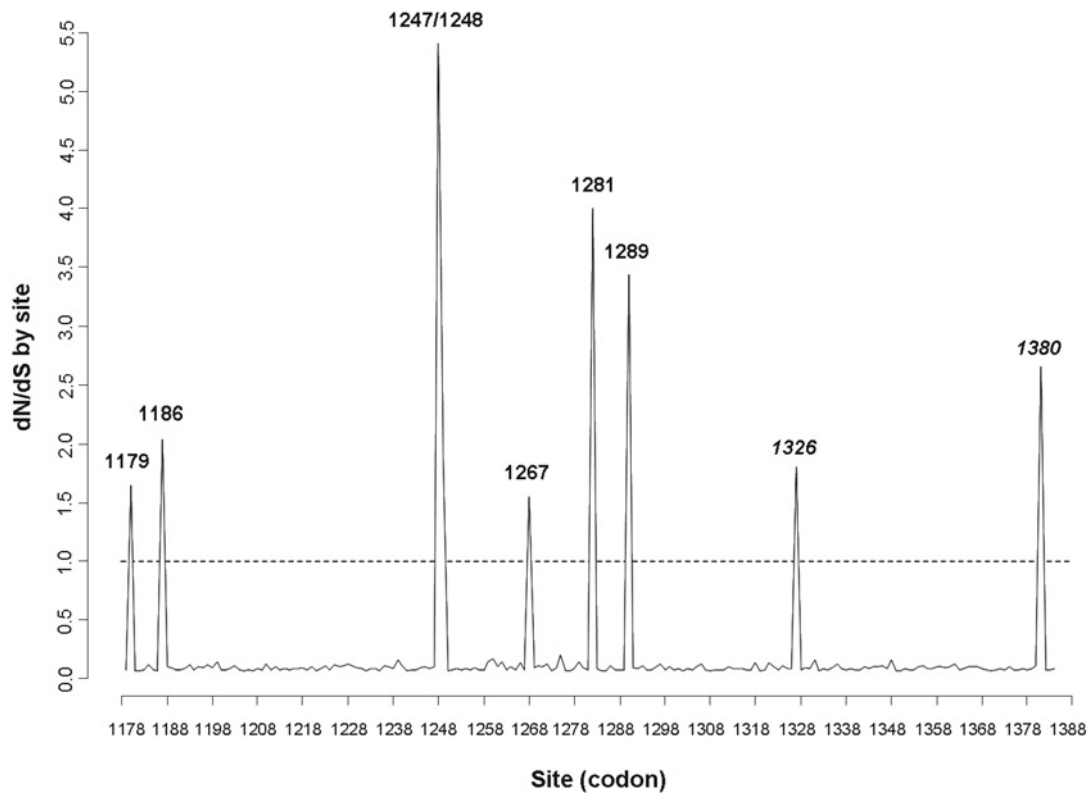


Figure 7: A Bayes Empirical Bayes graph showing dN/dS scores for 206 aa sites in the S protein derived from S gene fragments obtained from spotted hyenas. Peaks above a dN/dS value of 1 (dashed line) indicate sites that are under positive evolutionary selection while those below the line are under purifying selection. In bold: non-conservative aa substitutions at selected sites; in italics: conservative substitutions. Sites 1247 and 1281 were sites where both non-conservative and conservative substitutions occurred.

3.1.4 DISCUSSION

Results presented in this Chapter reveal considerable genetic diversity in coronaviral M gene and S gene fragments recovered from spotted hyena faeces collected from known individuals in three large clans in the Serengeti National Park and from one silver-backed jackal that inhabited the same area. Faeces collected during a relatively short monitoring period revealed five distinct CoV strains based on M gene fragments and at least four strains based on S gene fragments. Hyenas were infected with different CoV strains in different years suggesting either rapid virus evolution within this host species and/or the transmission of CoV strains to spotted hyenas from other host species. The detection of two distinct M gene fragments in one faecal sample from one hyena provided evidence for simultaneous infection of an individual with both a FCoV-II like and a CCoV-II like strain. Such co-infections provide potential for recombination events and may lead to the emergence of new CoV types (Herrewegh et al. 1998; Wesley 1999; Escutenaire et al, 2007; Decaro et al. 2009). A jackal that ranged within the territory of a hyena study clan was infected in 2007 with a CCoV-II strain distinct from the prevalent strain that infected all three hyena clans in that year and also from strains that

infected hyenas in all previous years, suggesting a degree of virus-host adaptation and species specificity. Interestingly, the feline-like CoV obtained in 2004 from a hyena was similar to FCoV-II strains, which are significantly less prevalent in domestic cat populations (10-20% of field isolates) than type-I strains (Benetka et al. 2004; Duarte et al. 2009).

CoVs are thought to exhibit a degree of host species specificity mostly mediated by adaptations in the S protein to host cell receptors used for virus attachment and entry (Kuo et al. 2000; Lai & Holmes 2001). The use of different host cell receptors by a specific CoV strain may permit infection of taxonomically diverse host species (Yeager et al. 1992; Li et al. 2003; Hofmann et al. 2006; Dye et al. 2007). My results suggest that spotted hyenas and perhaps other species belonging to the Hyaenidae are susceptible to both FCoV-II-like and CCoV-II-like CoVs. This may indicate that spotted hyena cells present one receptor type that permits attachment and entry of FCoV-II like strains, and another receptor type that serves the same function for CCoV-II like strains. However, as the S gene of FCoV-II is thought to be closely related to the S gene in CCoV-II following a homologous recombination event, separate receptors for entry of FCoV-II-like and CCoV-II-like viruses may not be required. I currently do not know which of these possibilities explains the infection of hyenas with both FCoV-like and CCoV-like strains.

A key factor influencing the fitness of any particular CoV strain is its rate of transmission within a host population. Transmission requires contact between infected and susceptible hosts and the availability of susceptible animals will depend on the level of herd immunity (Anderson & May 1991; McCullum et al. 2001). The adult hyena population in the Serengeti National Park has a high seroprevalence of antibodies against CoV (between 68 % - 74 %; East et al. 2004; see also Chapter 3.4). Results of this Chapter suggest that CoV infection in hyenas was chiefly maintained in young susceptible cubs gathered at communal den sites inside the territory of each clan. CoV strains with fast transmission rates that outstrip the birth of naive, susceptible cubs would be prone to extinction when herd immunity in the adult population was sufficient to prevent virus transmission between spatially separated groups of susceptible cubs within group territories (see also Chapter 3.4). My results suggest that the CoV genotypes that infected hyenas in 2004 may have been extinct by 2007, but I cannot be certain of this.

How divergent do CoV strains need to be, to be unaffected by herd immunity to previous strains? Evolution of a new strain sufficiently divergent from existing strains may have an advantage if it can evade herd immunity and more easily spread between groups. This scenario may explain the presence of an FCoV-II like strain in 1997 in one of the hyena clans

monitored during this study (see East et al. 2004) and the presence of an FCoV-II-like strain in 2004, followed by the apparent replacement of FCoV-II-like strains by CCoV-II-like strains by 2006 and 2007. If correct, this scenario implies the regular extinction of CoV strains and the emergence of new strains adapted to hyenas that spread through the population of approximately 5300 spotted hyenas in the Serengeti National Park (Hofer & East 1995). New CoV strains might arise through rapid evolution within the hyena population and/or through the introduction of new strains from other reservoir species. Why spotted hyenas in 2004 were infected with divergent CoV strains, whereas in 2007 numerous individuals were infected with an identical strain is unclear. The high prevalence of this dominant strain and its presence in all study clans in 2007 suggests it had successfully spread through the Serengeti spotted hyena population.

CoVs are capable of rapid evolution and adaptation to new host species through point mutations, and small insertions and deletions in coding and non-coding regions of the viral genome (Laude et al. 1993; Vennema et al. 1998; Kuo et al. 2000; Lai & Holmes 2001; Erles et al. 2003; Buonavoglia et al. 2006). For example, deletions in the SARS-CoV ORF 8a/b gene may have resulted in the adaptation of SARS-CoV to civet cats and humans after the virus had been transferred from the natural reservoir, probably bats (Gorbalenya et al. 2006; Janies et al. 2008). The high mutation rate of CoVs during replication (Jenkins et al. 2002) suggests that rapid viral evolution may in part explain the viral diversity found during the few years of monitoring of this research.

My results provide some evidence for the adaptation of CoV strains to specific host species. I found that all aa sequences of S gene fragments from hyenas in 2007 were identical and contained a unique non-conservative substitution (Fig. 5) and presented a hydrophobicity profile that differed from all other (Fig. 6). In contrast, the CoV S gene fragment from the strain that infected a jackal in 2007 had a relatively low similarity (Table 3) in terms of its aa sequence and hydrophobicity profile (Fig. 6e) in comparison to the CoV strain that infected hyenas in 2007 (Fig 6d). The similarity between the hydrophobicity profile of the S gene fragment obtained from one hyena in 2006 (Fig 6c) and that of the fragment from a jackal in 2007 (Fig 6e) may indicate that both hyenas and jackals were infected by similar strains in 2006. Because CoV Hyena 06 was not detected in any hyenas in 2007, it is possible that the 2006 strain no longer infected hyenas, and it might be that selection on the 2006 hyena strain during transmission through the immune systems of hyenas led to the emergence of the prevalent hyena strain in 2007. Virus-host adaptation was also suggested by a previous study of a more variable fragment (5' region) of the S gene that revealed 20 unique substitution sites and a high

level of divergence to other CoV (East et al. 2004). The high similarity (99.5 %, Table 3) of an S gene fragment obtained from a jackal to published fragments from CCoV-II strains infecting domestic dogs suggests adaptation of the CCoV-II S protein to wild and domestic members of the Canidae.

The presence of the same unique aa substitution in the M gene fragment at position 121 in all sequences obtained from hyena hosts in 2006 and 2007 (Fig. 3) suggests that the strain that infected spotted hyenas in 2007 evolved from the strain present in this host population in 2006. Phylogenetic analyses of nt sequences in the M (Fig. 2) and S gene fragments (Fig. 4a) also supported this suggestion. Additionally, similarities in the aa sequences of S gene fragments provided some evidence of a similar evolutionary response to selection in strains from hyena hosts in 2004 and 2007 or that these strains may have a common origin (Fig. 4b).

Young spotted hyenas were significantly more likely to be infected with CoV than adults, an age-specific pattern of infection similar to that reported for CCoVs (Pratelli 2006). As I only obtained faeces from adult jackals and foxes, the lack of faecal samples from young of both species probably explains why only one CoV strain was obtained from both these species. More extensive screening of faeces from the young wild carnivores will most likely reveal an even greater diversity of CoV strains in the carnivore community in the Serengeti National Park. The phenomenon that none of the samples obtained from hyenas inhabiting the adjacent Ngorongoro Crater were infected with CoV is examined and discussed in Chapter 3.4.

In summary my results suggest a moderate degree of species specificity, considerable genetic diversity and temporal change in prevailing CoV strains that infect African wild carnivore species. This indicates that knowledge of the dynamics of CoV diversity, evolution and extinction within a socially and spatially structured group-living carnivore requires long-term monitoring that includes key sympatric species.

3.2 Detection, characterisation and prevalence of calicivirus-like variants in free-ranging spotted hyenas

3.2.1 INTRODUCTION

The Caliciviridae is a highly variable family of small, non-enveloped viruses with single-stranded, positive RNA genomes of 7.4 – 8.3 kb with considerable antigenic and genetic diversity between and within genera (Green et al. 2001). All positive-stranded RNA viruses encode a RNA-dependent RNA-polymerase (RdRp). The RdRp is an enzyme that catalyses the replication of RNA from an RNA template (Buck 1996; Lai 1998). The complementary strand is able to act as a template for the production of new virus genomes which are further packaged and released from the cell ready to infect more host cells. All RNA viruses share high mutation rates due to the lack of proof-reading repair mechanisms of viral RdRps (Steinhauer et al. 1992; Domingo & Holland 1994). This error-prone replication leads to a high mutation rate and therefore high genetic diversity and creates a substantial potential for rapid evolution that allows RNA viruses to exploit new niches such as new host species (Holland et al. 1982; Domingo & Holland 1994; Domingo et al. 1997). This high genetic diversity of RNA viruses contributed also to the emergence of highly transmissible globally distributed strains of human caliciviruses (CVs) (Noel et al. 1999) as well as some virulent animal CVs that can frequently be lethal (Pederson et al. 2000; Moss et al. 2002; Coyne et al. 2006).

The Caliciviridae include four genera: Vesivirus, Lagovirus, Norovirus and Sapovirus (Berke & Matson 2000; Green et al. 2000; Buchen-Osmond 2003), and have a broad host range. First observed in pigs and domestic cats, caliciviruses were later also found in reptiles, rodents, felines, canines, birds, marine mammals (Smith et al. 1998), chimpanzees and humans (Green et al. 1995; Seal and Neill 1995; Berke et al. 1997; Lamarque et al. 1997; Fankhauser et al. 1998; Liu et al. 1999). CVs furthermore have the ability to cross species barriers (Lamarque et al. 1997; Smith et al. 1998). Current knowledge on molecular characteristics of CVs is mainly confined to CVs infecting humans and animals such as domestic cats (Radford et al. 2007), domestic dogs (Martella et al. 2008b), mink (Guo et al. 2001) and swine (Sugieda et al. 1998; van der Poel et al. 2000; Wang et al. 2005).

CVs infecting humans can be found in two genera: sapoviruses (SaVs) and noroviruses (NoVs), previously known as ‘Norwalk-like viruses’ and ‘Sapporo-like viruses’. These viruses are regarded as a major cause of epidemic acute gastroenteritis since Norwalk virus was detected as the cause of an outbreak of gastroenteritis in Norwalk, Ohio, in 1972 (Kapikian et

al. 1972) and the prototype Sapporo virus was detected as the cause of a similar outbreak in an infant home in Sapporo, Japan, in 1977 (Chiba et al. 1979). Recently, enteric porcine NoVs and SaVs similar to human NoVs and SaVs were detected in pigs, raising questions of whether humans serve as reservoirs for transmission of CVs to pigs and what role pigs may play as reservoirs for the emergence of human NoVs and SaVs (Guo et al. 1999; Farkas et al. 2005; Wang et al. 2005; 2006; Martella et al. 2008a). SaVs were detected in all age classes of pigs irrespective of whether individuals did or did not show symptoms of diarrhoea, with a low prevalence in nursed piglets possibly owing to protection of maternal antibodies in the milk. Prevalence of SaV shedding in adult sows was high. The highest prevalence was observed in post-weaned piglets probably because during this period piglets did not receive maternal antibodies through milk anymore and were under social and environmental stress (Wang et al. 2006). Recently a novel CV genetically related to human NoVs was detected in a captive lion cub that died of severe hemorrhagic enteritis in Italy, suggesting another species jump from humans to animals (Martella et al. 2007).

CVs infecting non-human mammals have been found in the genera (1) *Lagovirus* which includes rabbit hemorrhagic disease virus (RHDV) (Liu et al. 1984; Ohlinger et al. 1990) as well as the European brown hare syndrome virus (EBHSV) (Nowotny et al. 1997), and (2) *Vesivirus* which includes vesicular exanthema of swine virus (VESV) (Cubitt et al. 1995), San Miguel sea lion virus (SMSV) (Smith et al. 1973), mink calicivirus (MCV) (Guo et al. 2001), feline calicivirus (FCV) (Gaskell et al. 2006) and canine calicivirus (CaCV) (Mochizuki et al. 1993, Matsuura et al. 2002; Martella et al. 2008b).

Most information on CV infection in domestic carnivores comes from studies of domestic dogs and cats. In domestic cats, FCV is a highly infectious respiratory and oral pathogen (Gaskell et al. 2004) typically with a high prevalence in the domestic cat population, with higher levels recorded in larger social groups than in small groups with only few cats (Binns et al. 2000; Helps et al. 2005). Domestic cats may consistently shed FCV over long periods of time, virus shedding may be intermittent, and some cats never shed virus and may be resistant to infection (Coyne et al. 2006). Although recognised strains of FCV are usually not associated with significant mortality, the calicivirus genome is highly mutable and more virulent strains that are lethal can arise at any time (Pederson et al. 2000; Schorr-Evans et al. 2003; Hurley et al. 2004; Coyne et al. 2006). The first CV in a domestic dog was isolated from the faeces of a 4-year-old domestic dog with bloody diarrhoea and clinical symptoms indicating a disturbance to the central nervous system in Tennessee, USA, in 1985 (Schaffer et al. 1985). In 1990, another CV was identified in Japan in a 2-month-old pup with intermittent diarrhoea

(Mochizuki et al. 1993). The virus was antigenically and genetically unrelated to FCV and was classified as a CaCV in the Vesivirus genus (Matsuura et al. 2002). A CaCV has also recently been detected in a domestic dog with diarrhoea in Italy (Martella et al. 2008b).

Most information on CV infection of wild carnivores consists of serological surveys. Antibodies to FCV have been reported in lynx *Lynx canadensis* (Biek et al. 2002), bobcats *Lynx rufus* (Fox 1983) and wild cats *Felis sylvestris sylvestris* (Artois & Remond 1994; Daniels et al. 1999; Leutenegger et al. 1999). Brazilian pumas *Puma concolor*, ocelots *Leopardus pardalis* and little spotted cats *Leopardus tigrinus* also showed evidence of infection with FCV (Filoni et al. 2006). Antibodies to FCV have also been reported in free-ranging lions in the Serengeti ecosystem whereby exposure was high (82%, n = 255) in lions inhabiting the Serengeti National Park and low (2%, n = 51) in lions inhabiting the Ngorongoro Crater (Hofmann-Lehmann et al. 1996; Packer et al. 1999). There is also evidence of CV infection of spotted hyenas in the Maasai Mara National Reserve in the northern, Kenyan part of the Serengeti ecosystem (Fig. 1, Chapter 2). Antibody prevalence in juvenile and adult hyenas was similar and did not vary across years, suggesting that CV infection was endemic there (Harrison et al. 2004).

To my knowledge, there has been no molecular characterisation of CV strains circulating in free-ranging African wild carnivores. As caliciviral RNA was obtained from faecal samples from dogs (Martella et al. 2008b) using reverse transcriptase PCRs (RT-PCRs) I applied this non-invasive method to survey CV infection in a large number of individually known spotted hyenas from a metapopulation of spotted hyenas in the Serengeti ecosystem, consisting of hyenas in the Serengeti National Park and the adjacent Ngorongoro Crater. To investigate whether hyenas shed CV in their faeces I initially used a broadly reactive primer pair targeted to highly conserved motifs of the RdRp region (Jiang et al. 1999). Currently, there are eight conserved motifs identified in caliciviral RdRps and the two motifs covered by the primer pairs I used are suggested to play an important role in RdRp activity (Poch et al. 1989; Koonin 1991).

The aim of this study was to identify CV variants infecting spotted hyenas in East Africa during a period of several years of non-invasive monitoring of CV excretion in faeces of several hundred individually known animals and establish their phylogenetical relationships to known variants. I expected the genetic type of CV infecting the spotted hyena to be most closely related to either feline or canine CVs, as this was found in infections with other viruses (East et al. 2001, 2004; see also Chapter 3.1). I compared CV variants infecting the two subpopulations within approximately the same general time-frame. These subpopulations were to some

extent separated by the physical barrier formed by the rim of the Ngorongoro Crater and differed to some extent in their ecological and demographic profiles (for details see Chapter 2). I therefore investigated whether prevalence of infection differed between both subpopulations. Furthermore, I examined whether patterns of infection were influenced by demographic and ecological parameters such as age, sex, prey availability and social status. I expected that infection prevalence in both subpopulations should be higher in young, susceptible individuals than in older animals. Because of the strong effect of social status in spotted hyena society on access to food resources in a clan territory I expected infection to be higher in individuals of lower social status as they usually have a lower nutritional status than animals of higher social status (Hofer & East 2003). To my knowledge, this is the first study to genetically characterise CV variants and infection patterns in a free-ranging wild carnivore species in Africa.

3.2.2 METHODS

Study animals

In this Chapter I included data obtained from three spotted hyena clans in the Serengeti National Park and all eight clans resident on the Crater floor. From the three clans inhabiting the Serengeti National Park I screened altogether 229 faecal samples collected immediately after deposition between 1995 and 2007 from 213 individually known spotted hyenas (119 cubs and 94 individuals older than 12 months) and blood and tissue samples from 85 individuals including 38 blood samples and 47 tissue samples (details on sort of tissues see Appendix A). From the eight clans inhabiting the Crater I screened 86 faecal samples from 82 individuals (42 cubs and 40 older than 12 months of age) and tissue samples from 19 individuals collected between 1997 and 2006. I obtained multiple faecal samples from three individual Serengeti hyenas to approximately determine the viral shedding period. I also screened successive faecal samples from seven Serengeti individuals after initial infection during periods spanning six months to four years to test whether initial CV infection might induce life-long immunity.

RNA isolation and RT-PCR

Viral RNA was isolated from samples stored and transported as described in Chapter 2. For initial screening of samples and molecular characterisation of CVs obtained from spotted hyenas I initially used a previously published broadly reactive primer pair (p289 and p290) that targeted the highly conserved aa motifs “DYSKWDST” and “YGDD” of the RdRp gene of CVs (Jiang et al. 1999) and amplifies a 285 nt fragment. After sequencing positive samples, I

designed a more specific hyena CV primer pair termed CaliF2 (5'-CAG TGA CAG CCA CAT CCT TG-3') and CaliR2 (5'-AGC ACT GCA GCA GCA AAG TA-3') that amplified a 208 nt long fragment of the RdRp gene. I performed the RT-PCRs as described in Chapter 2 with a primer annealing temperature of 55°C. I considered samples that showed bands of the expected length with both primer pairs as positive, and ran all PCRs in duplicate to ensure that results were reliable.

Sequencing of PCR products and phylogenetic analysis

I sequenced PCR amplification products obtained from 15 infected spotted hyenas (13 samples from Serengeti spotted hyenas, including 12 faecal samples and one lung sample; two faecal samples from Crater spotted hyenas). Sequences generated in this study and used for phylogenetic analyses were tagged with the individual animal's identity (ID), the date (year, month, day) of sample collection and SNP if from the Serengeti National Park subpopulation (e.g. P412/071001/SNP), and NC if from the Ngorongoro Crater subpopulation and are contained in Appendix C. The sequence obtained from the lung sample was additionally marked with '_lu' after the animal ID. I edited and analysed the sequences as described in Chapter 2. Phylogenetic trees were constructed by the NJ method using Tamura Nei parameter for nt alignments and a Jones-Taylor-Thornton (JTT) matrix for aa alignments as implemented in MEGA 4.0 (Tamura et al. 2007).

Statistical analysis

To test for differences of prevalence of infection between the Serengeti and Crater hyena subpopulations I applied log-likelihood ratio chi-square tests. I also applied this test to determine differences in prevalence of infection across years in both subpopulations. To determine factors that may influence viral shedding frequency I performed binomial logistic regression models that included as effect variables age, sex, social status and prey availability. Only individuals for which data on all factors were available were included in the statistical analysis.

3.2.3 RESULTS

Prevalence

a) Prevalence of infection in the Serengeti and Crater population

Of 213 faecal samples from individuals collected between 1995 and 2007 in the Serengeti subpopulation I detected caliciviral RNA in faeces from 111 individuals (52 % positive) including 72 of 119 cubs (61 % positive) and 39 of 94 individuals (41 % positive) older than 12

months. Of 111 positive tested individuals eight showed clinical signs such as coughing (one adult, two cubs) green nasal discharge (two cubs), and signs of conjunctivitis (discharge and swollen eyes; three cubs). Of 82 Crater hyenas screened between 1997 and 2006, I detected caliciviral RNA in only 18 individuals (22 % positive) including 10 of 42 cubs (24 % positive) and 8 of 40 (20 % positive) individuals older than 12 months (Table 4). Caliciviral RNA was significantly more often present in faeces from hyenas from the Serengeti subpopulation than in the faeces from hyenas inhabiting the Crater (log-likelihood ratio $\chi^2 = 23.75$, d.f. = 1, n = 295, $p < 0.00005$). Of the 38 blood samples from the Serengeti hyena subpopulation none was positive; of the 47 tissue samples one lung sample was positive. None of the 19 tissue samples from the Crater hyenas were positive.

b) Parameters influencing infection status

As shedding prevalence in the Serengeti and Crater hyena subpopulation differed significantly I applied binomial logistic regression models separately for both subpopulations. The binomial logistic regression model for the Serengeti hyena subpopulation ($G = 28.4$, d.f. = 5, n = 203, $p < 0.0001$) revealed that cubs were more likely to shed virus than individuals > 12 months of age ($Z = 2.45$, d.f. = 1, $p = 0.014$) and lower ranking individuals were more likely to shed virus than higher ranking individuals ($Z = 2.36$, d.f. = 1 $p = 0.018$). The likelihood of shedding was higher during times of high or low prey abundance than during times of medium prey abundance ($Z = -3.60$, d.f. = 2, $p < 0.0001$). Sex had no influence on the likelihood of virus shedding ($Z = 1.34$, d.f. = 1, $p = 0.181$). In contrast, the binomial logistic regression model for the Crater hyena subpopulation was not significant ($G = 9.24$, d.f. = 5, n = 81, $p = 0.18$), suggesting that none of the above parameters influenced the likelihood of virus prevalence in the Crater subpopulation.

c) Prevalence of infection across years

As virus shedding differed between cubs and individuals older than 12 months of age I tested variation of shedding prevalence across years separately for cubs and individuals > 12 months of age in the Serengeti population. In the Crater, the likelihood of virus shedding was similar in cubs and older individuals and thus I investigated CV prevalence across years using data from all individuals. For statistical analysis only years with a sufficient sample size ($n \geq 5$) were included (Table 4).

Table 4: Prevalence of CV in faecal samples across years in the Serengeti (SNP) and Crater (NC) hyena sub-populations. Absolute number of positively tested individuals and number of samples tested each year are given. Percentages of positively tested hyenas are given below absolute numbers.(cub: individuals < 12 months of age, ad: individuals > 12 months of age).

Population	Age	1995	1997	1998	1999	2000	2001	2002	2003	2004	2005	2006	2007	Total
SNP	cub	1/1	1/1	-	-	-	3/3	4/6*	12/16*	14/15*	8/10*	19/33*	15/34*	72/119
		100	100				100	67	75	93	80	58	44	61
	ad	-	0/1	1/1	-	-	0/13*	2/3	4/4	1/1	7/19*	8/17*	16/35*	39/94
			0	100			0	67	100	100	37	47	46	41
NC	cub/ ad	-	1/2	0/1	1/9*	1/8*	-	1/4	2/12*	7/28*	3/13*	2/5*	-	18/82
			50	0	11	13		25	17	25	23	40		22

*: data included in statistical analysis

-: no data available

In the Serengeti subpopulation, the likelihood of viral excretion by cubs varied across years (log-likelihood ratio $\chi^2 = 15.4$, d.f. = 5, $n = 114$, $p = 0.009$). Between 2003 and 2005 reasonable sample sizes indicated a high level of infection (75-93%) followed by a decline in the prevalence of infection in 2006 and 2007. In the years before 2003, interpretation of results is not possible owing to the small sample size (Table 4). The likelihood of virus excretion of Serengeti individuals older than 12 months also varied across years (log-likelihood ratio $\chi^2 = 13.8$, d.f. = 3, $n = 84$, $p = 0.02$) with no evidence of infection in 2001, evidence of infection in the small number of samples screened between 2002 and 2004 and evidence of infection between 2005 and 2007 ranging from 37% to 47%, thus below the infection prevalence in cubs in those years. In the Crater population shedding prevalence did not vary across years (log-likelihood ratio $\chi^2 = 2.4$, d.f. = 5, $n = 75$, $p = 0.8$) showing similar moderate levels between 1999 and 2006 (11% to 40% infected, Table 4).

d) Screening of subsequent samples

All seven Serengeti individuals of which multiple CV positive samples were collected demonstrated virus shedding in successive faecal samples after the first positive sample was detected (Table 5). Information is not sufficient to establish if this was a consequence of long-term persistence, intermittent viral shedding or a succession of re-infections.

Table 5: Multiple CV positive tested faecal samples from seven spotted hyenas in the Serengeti National Park.

Animal ID	date tested positive	days after first positive sample	years after first positive sample
I366	20021229	0	
	20030710	193	0.53
P399	20030701	0	
	20040201	215	0.59
	20051231	699	1.92
P420	20040219	0	
	20050620	487	1.33
M297	20060304	0	
	20071019	594	1.63
I403	20050623	0	
	20070507	683	1.87
P412	20040223	0	
	20071001	1316	3.61
I383	20040121	0	
	20071022	1370	3.75

Phylogenetic analysis

I obtained seven 285 nt long fragments of the caliciviral RdRp region from the Serengeti hyena population. In addition I obtained eight shorter fragments of 208 nt length including two sequences from the Crater population and six sequences from the Serengeti population (five of these sequences were from faecal samples and one sequence was from a positive lung sample).

Based on a 208 nt and 285 nt alignment (Fig. 8a) all CV sequences from spotted hyenas in both the Crater and the Serengeti, formed a tight cluster and were distinct from known CVs infecting domestic dogs and cats. Phylogenetically, the CV sequences from spotted hyenas were most closely related to human SaVs and SaVs of pigs but still distinct from both human and pig SaVs. Based on the deduced aa sequences, all short and long sequences obtained from hyenas were identical and again a strong cluster of the hyena CVs separate from published sequences was identified (Fig. 8b).

Comparison of the nt sequence similarities of all caliciviral sequences obtained from spotted hyenas revealed that the RdRp sequences were variable and showed similarities ranging from 86.5 % to 100% (Table 6). Serengeti sequences (285 nt) P418/040221/SNP and P420/040219/SNP were identical and grouped as ‘hyena 1/SNP; the Serengeti sequences (208 nt) M425/020717/SNP, I403/070507/SNP and I459/071108/SNP as well as both Crater sequences E085/021219/NC and A056/030811/NC were also identical and grouped as ‘hyena 2/SNP-NC in Table 6. Comparison of the fragments obtained from spotted hyenas with pub-

lished sequences revealed relatively low similarities with all published sequences. The highest similarity of CV variants obtained from spotted hyena hosts using a sequence of 285 nt ('hyena 1/SNP' and I422/060215/SNP) was 66.6 % to a human SaV detected in Mexico in 1990 (S._hu/MEX/AY157867) and similarities of other sequences of the respective length obtained from hyenas to a SaV obtained from a human in Germany (S. hu/GER/EF064148) ranged from 65.2 – 66.3% (Table 6). When considering the short sequences (208 nts), the highest similarity of hyena sequence P412/071001/SNP was obtained to a SaV detected in a human in Germany (S. hu/GER/EF064148) in 2002 (69.7%, Table 6). This sequence from a hyena host in 2007 was the most divergent from all other sequences obtained from spotted hyenas with similarities ranging from 86.5 to 88.4% and was also placed basal to all other sequences in the nt and aa trees (Fig. 8a, b). Similarities of other sequences of the respective length obtained from hyenas to a SaV from a human in India (S. hu/IND/AB447416) in 2007 ranged from 67.7 – 68.2%. Comparison of the fragments obtained from hyenas to published caliciviral sequences detected in domestic pigs revealed relatively low similarities ranging from 53.6% to 60.7% and lowest similarities of CV hyena sequences were determined in comparison to published CV variants infecting domestic dogs and domestic cats ranging from 43.8% to 50.0%.

Comparison of all CV sequences obtained from spotted hyenas illustrated that most nt substitutions occurred when comparing the spotted hyena CVs to other published CVs detected in humans, domestic pigs, domestic dogs and domestic cats but nt substitutions were also observed between CV spotted hyena sequences (Table 6).

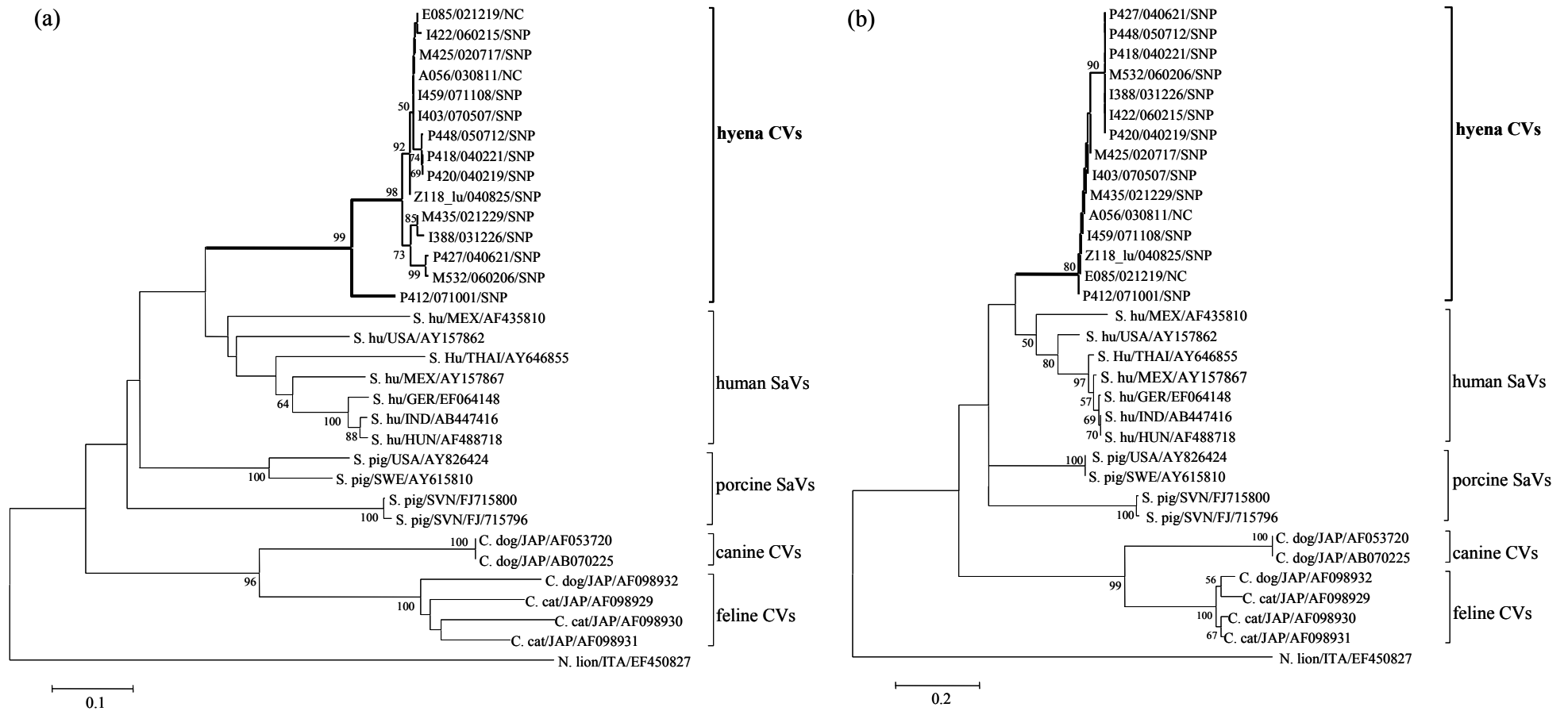


Figure 8: Phylogenetic relationship between CVs from spotted hyenas and published human and porcine SaVs, feline and canine CVs. A NoV from a captive lion served as out-group.

(a) NJ tree based on 208 and 285 nt fragments of the RdRp gene and a Tamura Nei parameter. Scale bar indicates the estimated number of nt substitutions per site. (b) NJ tree based on 80 and 95aa fragments of the RdRp gene using JTT matrix. Numbers at nodes represent the percentage of 1000 bootstrap replicates. Scale bar indicates the estimated number of aa substitutions per site.

Table 6: Similarities of caliciviral RdRp fragments (208nt and 285nt) obtained from Serengeti ecosystem spotted hyenas to published human and porcine SaVs and CVs detected in domestic dogs, domestic cats and a captive lion. In bold: highest similarities of hyena CV sequences to published human SaVs.

Calicivirus variants	long sequences (285 nt)						short sequences (208nt)			
	Hyena 1 SNP ¹	I388 031226 SNP	P427 040621 SNP	P448 050712 SNP	I422 060215 SNP	M532 060206 SNP	Hyena 2 SNP-NC ²	M435 021229 SNP	Z118_lu 040825 SNP	P412 071001 SNP
CVs hyenas (285nt)										
Hyena 1/SNP ¹	100	95.4	95.0	99.6	98.9	95.0	99.0	96.6	99.5	87.9
I388/031226/SNP		100	97.1	95.0	95.0	97.1	96.6	100.0	97.1	88.4
P427/040621/SNP			100	94.7	94.7	99.2	96.1	97.1	96.6	86.5
P448/050712/SNP				100	98.5	94.7	99.0	96.6	99.5	87.9
I422/060215/SNP					100	94.7	100	96.6	99.5	87.9
M532/060206/SNP						100	96.1	97.1	96.6	87.0
CVs hyenas (208nt)										
Hyena 2/SNP-NC ²	99.0	96.6	96.1	99.0	100.0	96.1	100	96.6	99.5	87.9
M435/021229/SNP	96.6	100	97.1	96.6	96.6	97.1		100	97.1	88.4
Z118/040825/SNP	99.5	97.1	96.6	99.5	99.5	96.6			100	88.4
P412/071001/SNP	87.9	88.4	86.5	87.9	87.9	87.0				100
SaVs humans										
S. hu/GER/EF064148	65.9	65.9	66.3	65.6	65.2	66.3	67.3	68.2	67.7	69.7
S. hu/MEX/AY157867	66.6	65.6	65.6	66.3	66.6	65.6	66.8	67.7	67.3	69.2
S. hu/IND/AB447416	66.3	65.6	65.9	65.9	65.6	65.9	67.7	67.7	68.2	68.2
S. hu/HUN/AF488718	65.6	64.9	65.2	65.2	64.9	65.2	67.3	67.3	67.7	68.2
S. hu/USA/AY157862	65.2	64.2	64.2	64.9	65.6	64.2	65.8	65.8	66.3	66.8
S. hu/THAI/AY646855	61.0	59.6	59.6	61.4	60.7	59.6	61.0	60.5	61.5	60.0
S. hu/MEX/AF435810	61.0	60.0	59.6	60.7	60.7	59.2	61.0	61.0	61.5	62.5
SaVs pigs										
S. pig/SWE/AY615810	60.7	59.2	60.0	61.0	60.3	59.6	58.6	57.2	58.1	56.7
S. pig/USA/AY826424	59.2	58.5	58.9	59.6	58.9	58.5	56.2	55.2	56.7	57.2
S. pig/SVN/FJ715800	55.7	54.7	54.0	56.1	55.4	54.0	54.8	54.8	54.3	55.2
S. pig/SVN/FJ715796	55.4	54.3	53.6	55.7	55.0	53.6	54.3	54.3	53.8	55.7
CVs cats and dogs										
C. dog/JAP/AF053720& AB070225 ³	48.4	49.8	48.7	48.0	49.1	49.1	50.0	50.4	49.5	48.5
C. dog/JAP/AF098932	44.2	44.9	45.6	44.2	45.2	45.6	48.5	47.5	48.0	48.0
C. cat/JAP/AF098929	45.2	45.2	45.6	45.2	45.2	45.6	49.0	48.0	48.5	49.0
C. cat/JAP/AF098930	44.9	44.2	43.8	44.9	44.9	43.8	46.6	46.6	47.1	49.5
C. cat/JAP/AF098931	45.2	45.6	45.2	45.2	45.2	45.2	47.1	47.5	47.5	51.4
NoV captive lion										
N. lion/ITA/EF450827	41.3	41.6	41.6	41.3	41.3	41.6	25.3	25.3	25.8	26.3

¹ hyena 1/SNP: identical sequences (285nt) from the Serengeti hyenas P418/040221/SNP and P420/040219/SNP

² hyena 2/SNP-NC: identical sequences (208nt) from Serengeti hyenas M425/020717/SNP, I403/070507/SNP, I459/071108/SNP and Crater hyenas E085/021219/NC and A056/030811/NC

³ C. dog/JAP/AF053720 and C. dog/JAP/AB070225 were identical

All nt substitutions within the hyena sequences turned out to be synonymous as the translated aa sequence of a length of 65 aa in the short sequences and 95 aa in the long sequence were 100% identical. In contrast, the hyena CV sequences displayed many aa differences in comparison to other CVs from humans, domestic pigs, domestic dogs and domestic cats (Fig. 9). All aligned sequences contained the highly conserved aa motifs “GLPSG” and “NS” (indicated by boxes in Fig. 9).

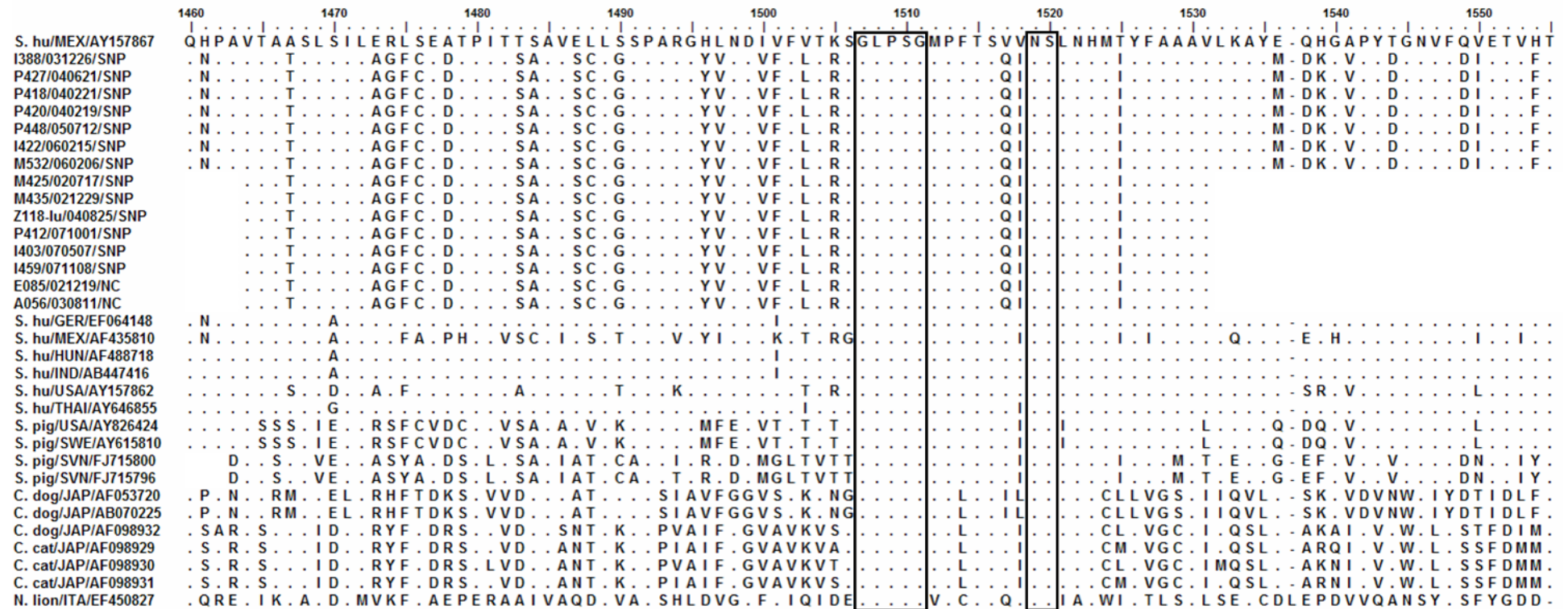


Figure 9: Alignment of the caliciviral RdRp gene fragments of 65 and 95 aa obtained from spotted hyenas and published data from human and porcine SaVs, and feline and canine CVs. Sequences were aligned using the human SaV ‘S.hu/MEX/AY157867’ as reference strain. Boxes indicate highly conserved motifs in the RdRp genome.

The highest similarity of the CV aa sequences obtained from the hyenas was 66.6% in comparison to the SaV strain S. hu/MEX/AY157867 that was obtained from a human in Mexico (Table 7). Similarities in comparison to strains detected in pigs ranged between 55.4% and 60.7% (Table 7). Lowest similarities were obtained when comparing the aa sequences of hyena CVs with CVs from dogs and cats and ranged between 40.0% and 41% (Table 7).

Table 7: Comparison of the caliciviral RdRp sequence (95aa) obtained from Serengeti ecosystem spotted hyenas with other published CaVs from humans, domestic pigs, domestic dogs and domestic cats. All caliciviral aa sequences from the hyena CVs were 100% identical and grouped as ‘CV hyenas’. In bold: highest similarity of hyena sequences to a human SaV detected in Mexico.

CV variants	CVs hyenas
CVs hyenas	100
SaVs humans	
S. hu/MEX/AY157867	66.6
S. hu/IND/AB447416	66.3
S. hu/GER/EF064148	65.9
S. hu/HUN/AF488718	65.6
S. hu/USA/AY157862	65.2
S. hu/THAI/AY646855	61.0
S. hu/MEX/AF435810	61.0
SaVs pigs	
S. pig/SWE/AY615810	60.7
S. pig/USA/AY826424	59.2
S. pig/SVN/FJ715800	55.7
S. pig/SVN/FJ715796	55.4
CVs cats and dogs	
C. dog/JAP/AF053720/AB070225	41.0
C. dog/JAP/AF098932	41.0
C. cat/JAP/AF098929	41.0
C. cat/JAP/AF098930	40.0
C. cat/JAP/AF098931	41.0
NoV captive lion	
N. lion/ITA/EF450827	28.1

3.2.4 DISCUSSION

To my knowledge, I demonstrate for the first time excretion of CVs in the faeces of a free-ranging African carnivore, the spotted hyena, using non-invasive RT-PCR methods. Molecular characterisation of the sequences obtained confirmed that caliciviral RNA is detectable in faecal matter from spotted hyenas. The previously published broadly reactive primer pair targeted to the highly conserved aa motifs “DYSKWDST” and “YGDD” in the RdRp gene of CVs (Jiang et al. 1999) yielded positive PCR results, indicating that these motifs are also conserved in CVs infecting spotted hyenas. These motifs are considered to be important for RdRp activity as single aa substitutions may inactivate RdRp functionality (Inokuchi & Hirashima 1987; Kroner et al. 1989). Analysis of the deduced 65/95 aa RdRp gene fragment revealed that all spotted hyena fragments contained the aa motifs “GLPSG” and “NS” which are highly

conserved in the RdRp gene of Caliciviridae (Hashimoto et al. 1999) and are suggested to be involved in substrate binding (Koonin 1991). These results demonstrate that the obtained sequences are indeed CV genomic fragments.

Eight of the positively tested individuals showed clinical signs of coughing, nasal discharge and conjunctivitis which are typical signs of respiratory FCV infection in domestic cats (Cai et al. 2002; Radford et al. 2007). However, I cannot say with certainty whether these clinical signs resulted from acute CV infection or from other possible secondary (for example bacterial) infections. None of the CV positive tested individuals showed clinical signs of gastroenteritis as is typical for SaV and NoV infection in humans (Kapikian et al. 1972; Chiba et al. 1979). The fact that viral RNA could be detected in a single tissue (lung) sample and the observed clinical signs may indicate respiratory infection. To find out whether CV in hyenas infects the respiratory or gastrointestinal tract, further monitoring of infected hyenas is required. However, none of the blood samples was tested positive, indicating that CV infection remains predominantly subclinical in spotted hyenas and rarely results in systemic infection.

Unexpectedly, the caliciviral RdRp sequences I obtained were less homologous to CVs infecting domestic cats and domestic dogs than to CVs infecting humans belonging to the genus Sapovirus (Table 4). The level of homology between CVs from spotted hyenas and humans was low, suggesting that spotted hyenas in the Serengeti ecosystem are infected with specific 'hyena' variants (see Fig. 8a, b). It remains to be established whether other carnivores in the ecosystem are infected with similar strain.

It is known for some CVs that they have the potential to jump from one host species to another, and recombination events between genetically related CVs have been frequently described (Reuter et al. 2005; Phan et al. 2007). Recently, porcine NoVs genetically and antigenetically related to human NoVs were detected in faecal samples from domestic pigs whereby one recombinant CV was identified (Wang et al. 2005). Martella et al. (2008a) observed a similar case where they detected a porcine CV in domestic piglets that was genetically related to human SaVs and identified a recombinational site between the porcine virus and human SaVs. It remains unclear whether animal SaVs have emerged over time in humans by direct species jumps or by exchange of genetic material via recombination events that occurred in co-infected domestic pigs with porcine and human CVs.

The relatively close phylogenetic relationship of CVs detected in hyena hosts in the Serengeti National Park and Ngorongoro Crater to human SaVs could be a result of interspecific transmission of human CVs to spotted hyenas. Before 1959, when the Serengeti National Park was

declared a national park and coincidentally settlement, hunting and cultivation of landscape were prohibited, the Serengeti ecosystem was inhabited and utilised by nomadic Maasai pastoralists. In 1959, the Maasai were moved to the Ngorongoro Conservation Area (see Fig 1, Chapter 1). Thus, before 1959, contact of spotted hyenas with human faeces or contaminated waste was very likely. Furthermore, Maasai living a traditional life have no burial ceremony, and human corpses were left behind to be consumed by scavengers, thus making them accessible to spotted hyenas (Eames et al. 1987). This could have contributed to possible host species jumps of human CVs to spotted hyenas in former times. The high similarity between CVs obtained from Crater and Serengeti hyenas would then be a result of only short-term evolution from a common ancestral human CV strain that evolved and is still maintained in spotted hyenas in both subpopulations.

Today, spotted hyenas are frequent visitors to garbage dumps and other sources of scavengeable food, including cemeteries (Sutcliffe 1969, 1970) which may contribute to inter-specific transmissions of pathogens. Additionally, the Serengeti National Park and the Ngorongoro Crater are amongst the most popular tourist attractions in Tanzania. Several lodges and numerous campsites were established within the Serengeti National Park and on the Crater rim as well as in the Crater (campsites) over the past 40 years and are visited by tens of thousands of tourists each year. Jobs for local Tanzanians associated with the development of hotels and tourist camps has led to the expansion of staff villages close to hotels and the establishment of waste disposal sites where potentially contaminated waste accumulates in which spotted hyenas frequently rummage to search for food (own observations). Thus, contact of spotted hyenas with contaminated waste is likely to happen frequently and may thereby favour transmission of pathogens from humans to spotted hyenas, possibly contributing to the emergence of novel virulent pathogens.

Spotted hyenas in both the Serengeti and Crater subpopulations shed caliciviral RNA. The prevalence of CV infection varied significantly between subpopulations, with a substantially and significantly higher prevalence in the Serengeti than in the Crater subpopulation. Also, Serengeti hyena cubs were more likely to shed caliciviral RNA in their faeces than individuals older than 12 months of age. This age-specific pattern of infection is similar to patterns of SaV infection in humans in which infants and young children had a higher prevalence of infection than adults (Pang et al. 2000), although SaV-associated gastroenteritis outbreaks in adults have also been described (Noel et al. 1997). In contrast, I found no age-specific pattern of infection in the Crater subpopulation. How can this difference in prevalence be explained?

Firstly, this difference might be a consequence of differences in nutritional status. It is known that malnourished children in developing countries suffer more severely from CV infections than well-nourished ones (Patel et al. 2008). Serengeti hyena mothers regularly travel over long distances (40-70 km) in order to forage on the nearest large concentration of migratory herbivores when such herds are absent from their territories and thus they may be absent from their dependent cubs for two to nine days (Hofer & East 1993c). In contrast, higher prey densities in the Ngorongoro Crater allow mothers with dependent cubs to nurse their offspring on a daily basis and as a result, young animals below 12 months of age are in a better nutritional state (Wachter et al. 2002) and might devote more body reserves to immune function. In addition to nutritional challenges (see Hofer & East 1998), levels of sibling rivalry among Serengeti littermates are substantially higher than those among littermates in the Crater (Golla et al. 1999; Wachter et al. 2002) and elevated levels of stress, particularly glucocorticoids, may impair the immune function of Serengeti cubs. Therefore, cubs from the Crater subpopulation are typically in a better nutritional status than cubs from the Serengeti subpopulation. The good nutritional status of Crater spotted hyenas could explain lower levels of CV infections in the Crater subpopulation and a similar prevalence in both age groups. As outlined in Chapter 3.1, coronavirus infection was absent from the Crater subpopulation and this phenomenon is examined and discussed in further detail in Chapter 3.4.

Secondly, in the Serengeti National Park mean group size of spotted hyena clans is 45 adults and subadults and they defend large group territories (55.5 km²) throughout the year (Hofer & East 1993a), whereas mean clan size in the Crater is 22.3 adults and subadults and clans defend significantly smaller territories (26.6 km²) (Höner et al. 2005). Thus the number of cubs present at a communal den in the Serengeti is higher than in the Crater (see also Chapter 3.4). It is known from human SaVs and NoVs that the risk of infection with these viruses increases in places such as health care centres, infant/child day care centres and hospitals where an accumulation of many susceptible and infected people favours transmission rates (Hedlund et al. 2000; Akihara et al. 2005). In cats, prevalence of FCV infection also depends on group size, being higher in animal shelters with many domestic cats than in private households (Radford et al. 2001; Bannasch & Foley 2005; Helps et al. 2005). If CV infection in spotted hyenas follows the same rules of transmission, the larger number of cubs at communal dens in combination with a reduced health status in the Serengeti may explain the higher prevalence of infection in Serengeti cubs than in the Crater subpopulation. In addition, Serengeti hyenas were more likely to shed CV during times of high prey abundance when most members of a clan gather at the communal den as the social centre of the clan (East & Hofer 1991) and most

clan members frequently visit the communal den (Hofer & East 1993c). Thus, the increase in the number of contact partners and likely increase in contact rate during such periods might also favour CV transmission (Hofer & East 1993b; East & Hofer 2001).

In the Serengeti subpopulation, infection prevalence varied across years in both cubs and older individuals, suggesting epidemic waves of infection in the Serengeti, whilst in the Crater subpopulation no such fluctuations of infection prevalence across years was observed, suggesting an endemic situation in the Crater. The observed fluctuations in CV prevalence in Serengeti spotted hyenas are consistent with observations by Packer et al. (1999) who investigated exposure to FCV using serological data from lions in the Serengeti National Park and also observed fluctuations in seroprevalence in adults as well as immatures across years. The prevalence of antibodies also varied between Crater and Serengeti lions: only few (2%, $n = 52$) lions inhabiting the Crater showed antibody titres against FCV whereas 82% ($n = 255$) of lions from the Serengeti National Park were seropositive (Hofmann-Lehmann et al. 1996). It was suggested that limited rates of dispersal of lions between the Serengeti and Crater lion populations, because of the suboptimal habitat around the Crater rim, kept the spread of CV from the Serengeti to the Crater low (Hofmann-Lehmann et al. 1996). Seroprevalence of antibodies against FCV in spotted hyenas inhabiting the Maasai Mara National Reserve, the northern extension of the Serengeti ecosystem (see Fig. 1, Chapter 2), was high, prevalence was similar in juveniles and adults and there was no variation of seroprevalence across years, suggesting that infection in Maasai Mara spotted hyenas is endemic (Harrison et al. 2004).

However, the serological studies on lions and spotted hyenas do not permit the determination of the exact time point of infection but only indicate that the animals had been exposed to the virus at some point in time before the sample was taken. Exposure was tested by checking for the presence of antibodies against FCV. My phylogenetic analysis demonstrated that spotted hyenas carry CV variants distinct from FCV with their most closely related strains being human SVs. It is unclear at present whether the serological tests for antibodies against FCV would also identify antibodies against the spotted hyena variants identified in these studies. It therefore remains to be established whether the previous studies on lions and hyenas documented exposure to a different CV variant (because the spotted hyena variant from this study could not have been detected with serological tests for antibodies against FCV), or whether the serological test is sufficiently unspecific to cover a wide spectrum of antibodies.

The results obtained from successive samples from the same individual and the high prevalence of infection in adults also suggest that CV infection does not induce life-long immunity in spotted hyenas. Fluctuations in infection prevalence across years could be a result of waves

of infections in one year followed by a fade-out of immunity during the following time span and an increase in the proportion of susceptible animals that paves the way for the next wave of infection.

In the Serengeti spotted hyenas, individual social status influenced infection prevalence whereas in the Crater subpopulation no such effect was observed. Low-ranking Serengeti spotted hyenas were more likely to acquire CV infection than high-ranking individuals. Spotted hyenas in both the Crater and Serengeti subpopulations live in social groups with a similar social structure consisting of separate linear male and female dominance hierarchies (Hofer & East 1993a; Höner et al. 2005). A possible explanation of this differences between both subpopulations is that high fluctuations in prey abundance in the Serengeti induces low-ranking animals to embark on time-consuming, costly foraging trips during times of low and medium prey abundance because high-ranking spotted hyenas have priority of access to the food resources inside the clan territory (Hofer & East 2003) and therefore lower-ranking individuals show higher concentrations of faecal glucocorticoids indicating higher stress levels in these individuals (Goymann et al. 2001). In the Crater, high-ranking individuals also have priority of access to food, but hyenas of lower social status do not travel long distances to alternative sources and food, and their glucocorticoid levels are generally lower than those in the Serengeti (Goymann et al. 2001). Higher stress-levels may impair immune response to infection and probably contributed to increased infection prevalence in low ranking Serengeti spotted hyenas.

In summary, my results highlight differences in infection patterns between two subpopulations of a spotted hyena metapopulation, showing a higher prevalence of infection and age-specific prevalence in Serengeti spotted hyenas, in contrast to low prevalence and no age-specific pattern in the Crater spotted hyenas. Infection prevalence varied across years in the Serengeti subpopulation, consistent with the idea of epidemic waves but not in the Crater subpopulation where infection might be endemic. These differences are probably due to differences in demography (group size and associated contact opportunities) and habitat-specific patterns of prey fluctuations which necessitate very different, social status-dependent movement patterns and hence contact rates. The CVs obtained from spotted hyenas were distinct from previously known CVs and unexpectedly more closely related to human SaVs than to feline or canine CVs, suggesting inter-specific transmission of CVs from humans to spotted hyenas.

3.3 Life history, social and ecological parameters explain patterns of infection and co-infection with non-virulent pathogens and their fitness consequences

3.3.1 INTRODUCTION

Investigations on human diseases typically focus on highly infectious diseases such as measles, avian influenza or the recently emerged ‘swine influenza’. Childhood diseases such as measles and pertussis, primary cause of morbidity in the well nourished developed world, remain a major cause of mortality in developing countries (Walsh & Warren 1979). These infections in combination with concurrent infections such as malaria and viral or bacterial diseases still play a dominant role in age-specific patterns of mortality in many regions of the world (Bradley 1972). Field studies on the relationship between infection and life history parameters in free-ranging wildlife populations are scarce and often fail to address the temporal relationship between the occurrence and timing of infection and its effect on key life history traits such as survival or longevity. Most studies were carried out on single zoonotic pathogens such as rabies in red foxes *Vulpes vulpes* and domestic dogs (e.g. Páez et al. 2009) and brucellosis in domestic cattle, American bison *Bison bison* or North American elk *Cervus elaphus* (Dobson & Meagher 1996), or on pathogens that may be transmitted from wildlife to domestic stock such as bovine tuberculosis from Eurasian badgers *Meles meles* (Krebs et al. 1998).

Immunosuppression as a result of a single infection may make individuals more vulnerable to further infections with another pathogen (Graham et al. 2007; Cattadori et al. 2008) and exacerbate its consequences. In hosts infected simultaneously with more than one pathogen, pathogens may compete for host resources and such interactions may affect the fitness and virulence of pathogens involved. Co-infections can occur in various combinations of micro-parasites and macro-parasites. Micro-parasites are typically intracellular pathogens and include pathogens such as viruses, bacteria and protozoa with a rapid reproduction within a host and without a special infectious stage (see Hudson et al. 2002). Macro-parasites are typically extracellular pathogens and include parasitic species such as tapeworms (helminths) or arthropods where reproduction usually occurs via the transmission of free-living infectious stages that pass from one host to another (see Hudson et al. 2002). Extracellular and intracellular pathogens induce polarised immune responses involving the development of T helper (Th) cells with characteristic Th1 pro-inflammatory or Th2 anti-inflammatory cytokine profiles. The direction of the polarisation usually depends on the type of pathogen. Micro-

parasites induce Th1 responses, characterised by the production of pro-inflammatory mediators such as IL-12, IFN- γ and nitric oxide, macro-parasites induce Th2 responses characterised by the production of IL-4, IL-5 and IL-10 (Jankovic et al. 2001). A critical feature of these two types of responses is that they counter-regulate each other. If one type of immune response is stimulated, the other type may be suppressed, as shown in infected mice where a Th1 response inducing protozoan *Toxoplasma gondii* infection suppressed immune responses to helminth infection (Miller et al. 2009). Another example is infection of European rabbits *Oryctolagus cuniculus* with the immunosuppressive myxoma virus that enhanced the susceptibility of rabbits to infection by a gastrointestinal helminth (Cattadori et al. 2007). I would therefore expect that a juvenile hyena infected with one macro-parasite (helminth) or one micro-parasite (virus) is predisposed to acquire further secondary infections with another pathogen.

‘Stress’ is another factor likely to influence an individual’s susceptibility to infection. Stress can be generated by a wide range of disturbances, including environmental, anthropogenic and social factors (Sapolsky 1982; Hofer & East 1998; Goymann et al. 2001), and pathogens can also be viewed as agents with the potential of disrupting internal homeostasis (Toates 1995). The term ‘stress’ is commonly used to describe the physiological reaction of an individual to unpleasant or adverse conditions (Broom & Johnson 1993). Factors that generate ‘stress’ lead to a disruption of internal homeostasis, followed by behavioural or physiological adjustments that seek to reduce disruption and re-establish internal homeostasis. The adrenal cortex plays an important role in achieving this adaptive response, as it provides a hormonal response to disturbance of homeostasis by releasing glucocorticoid hormones to modify energy and protein metabolism and immunological reactions (Toates 1995; Hofer & East 1998). It is known that chronic exposure to ‘stress’ or intense ‘stress’ in social mammalian species, for example in spotted hyenas during periods of hierarchy formation or social instability, can result in elevated levels of glucocorticoids as a physiological ‘stress’ response (Sapolsky 1982; Saltzman et al. 1994; Goymann et al. 2001). This can result in negative physiological and pathological consequences, one of which is an impairment of the immune system (Toates 1995). For example, subordinate free-living baboons have a high basal cortisol secretion rate and therefore have fewer circulating lymphocytes than dominant baboons (Sapolsky 1990). Such a reduction in components of the active immune system is likely to result in an increased susceptibility to pathogen infection, morbidity or even mortality (Hofer & East 1998). Thus, stressful factors (stressors) that lead to prolonged secretion of elevated levels of glucocorticoids are likely to lead to an increase in an individual’s susceptibility to pathogens.

Long-term studies of pathogen infections in free-ranging wildlife populations are rare as are studies that examine the potential role of pathogens in determining lifetime reproductive success of individuals. Many pathogen infections remain subclinical in their hosts; this absence of obvious clinical progression of disease may lead to infections being overlooked or may be responsible for the assumption that the pathogen has no impact on host population dynamics. For example, there was evidence for reduced fecundity and longevity as well as higher infant mortality in a long-term study on simian immunodeficiency virus (SIV) infection in chimpanzees *Pan troglodytes* (Keele et al. 2009) which did not produce obvious clinical symptoms. I would therefore predict that a juvenile hyena infected with viruses whereby infection remains subclinical may experience a detrimental effect on its survival and longevity.

Many life history parameters such as age, sex, litter size in which an individual was raised and social status may influence an individual's susceptibility to acquire infections by modulating its immune response (Lee 2006; Martin et al. 2008; Martin 2009). In addition, food availability, social factors that influence an individual's access to food, and the degree of competition to gain access to food may also influence an individual's nutritional status and as consequence its susceptibility to infection (Gasbarre 1997; Beldomenico et al. 2008; Plowright et al. 2008). Host density and contact rate between susceptible and infected individuals can also influence pathogen transmission (Anderson & May 1991; Diekmann & Heesterbeek 2000).

In the following sections I will discuss key factors likely to influence susceptibility to infection in spotted hyenas. I derive predictions on how these factors are likely to influence infection and how they may be confounded with other factors.

Age and contact rates

Young individuals with a naïve immune system are generally more susceptible to infection and may suffer more severely from infections than older animals, as their immune responses to antigens are not yet fully developed. As shown in Chapter 3.1, young spotted hyena cubs in the Serengeti National Park less than 12 months of age were more prone to CoV infection than older individuals, and seroprevalence to CoV in this population increased with age (East et al. 2004, Chapter 3.1). As previously described, *Hepatozoon* infection in young hyenas was not necessarily benign and was the source of mortality in two out of 39 hyena cubs examined (5%; East et al. 2008; see also Chapter 3.5), whereas infection of adults was subclinical and is generally in most African wild carnivore species assumed to be benign. I would expect that young hyenas less than 12 months of age may be more susceptible not only to single infection with CoV or CV but also to co-infection with both pathogens.

Pathogen transmission often depends on the contact rate between infected and susceptible individuals within and between populations or groups of individuals, on the density of hosts and on population structure (Lindholm & Britton 2007; Webb et al. 2007). Pathogen transmission usually increases with host density and declines with a reduction in the number of hosts (Anderson & May 1991; Diekmann & Heesterbeek 2000; Hudson et al. 2002). In spotted hyenas, cubs are raised within the clan territory in a communal den for the first 12 months of life (Hofer & East 1995), resulting in high daily contact rates among cubs in communal dens (Höner et al. 2006). I would therefore expect that infection in young, susceptible cubs may increase with the number of cubs present at the communal den.

Sex

Sex-biased patterns of infection with macro-parasites were observed in some hosts. The biological mechanisms underlying sex-related differences in infection patterns are often divided into ecological and physiological mechanisms (e.g. Zuk & McKean 1996). In general, males appear to be more prone to parasitism than females (see Hudson et al. 2002). Behavioural differences between the sexes may expose males to ecological conditions where infection is more likely to occur. For example, male spadefoot toads *Scaphiopus couchii* spent more time immersed in ephemeral pools exposed to the parasite *Pseudodiploorchis americanus* than females who only visited these areas to lay their eggs (Tinsley 1989). Physiological mechanisms proposed to explain sex biases in infection include for example differences in the concentrations of hormones such as androgens (testosterone and others) or glucocorticoids. It is known that these hormones interact with the immune system and depress immune response (e.g. Klein 2000). Spotted hyena society is utterly female-dominated, with the top-ranking male socially subordinate to the lowest-ranking female (Hofer & East 1993). High social status provides fitness benefits and priority of access to food (Hofer & East 2003). I would therefore expect spotted hyena males to be more likely to acquire macro-parasites such as helminths as well as micro-parasites than female hyenas.

Nutritional and social status

In humans it is known that malnutrition and immunosuppression increase susceptibility to infectious diseases (e.g. Piyathilake et al. 2004) as nutrient limitations can lead to reduced immunocompetence (Gershoff et al. 1968; Jose & Good 1973; Gross & Newberne 1980). Evidence currently available from wildlife only consists of a few reports of associations between poor nutritional status and decreased survival owing to an increase in the probability of infection with pathogens (Beldomenico et al. 2008). In the Serengeti National Park, large fluctua-

tions of prey abundance alters the frequency at which hyena cubs are nursed, and thus alters the total level of maternal input. When high densities of migratory herbivores are absent from the clan territory, mothers – especially low-ranking females that have lower access to resident prey (Hofer & East 2003) – travel over long distances (up to 70km) in order to forage on the nearest concentrations of migratory prey (Hofer & East 1993a,b) and thus may be absent from their cubs for up to nine days (Hofer & East 1993c). Spotted hyena cubs are not weaned between 12 to 18 months of age and are strictly dependant on maternal milk for the first six months of their life; they do not accompany their lactating mother on foraging trips but remain at the communal den within the territory for the first 12 months of life (Hofer & East 1993c, 1995). During times of low prey abundance, when the migratory herds are absent from the territory, cubs are less frequently nursed and cub growth rates are lower during such times, indicating a poorer nutritional status (Hofer & East 1993c, 1997, 2008; Golla et al. 1999). If low maternal input leads to poorly nourished cubs I would expect that hyena cubs should be more susceptible to infection during periods of low prey abundance than during periods of medium or high prey abundance when the frequency of nursing is increased. As high-ranking mothers have priority of access to resident prey and thus more frequently nurse their cubs than low-ranking mothers I would expect that cubs from low-ranking mothers are more likely to become infected than cubs of high-ranking mothers.

Sibling rivalry in twin litters

Strong sibling rivalry with potentially fatal consequences has been reported in many avian (Mock 1984, 1987; Mock & Parker 1997; Drummond 2001) and some mammalian species (domestic pigs *Sus scrofa* by Fraser 1990; captive and free-ranging spotted hyenas by Frank et al. 1991; Hofer & East 1997; Golla et al. 1999). When siblicide nearly always occurs it is termed obligate siblicide, whereas when siblicide only occurs when food resources are rare it is termed facultative siblicide (Mock & Parker 1997). Avian models of facultative siblicide predict that when food availability is low, aggression rates between siblings and the incidence of siblicide should be high (e.g. Parker et al 1989; McNamara et al. 1994). In spotted hyenas, all females breed, producing litters of one, two or very rarely three cubs throughout the year (Hofer & East 1995, 2008; Wachter et al. 2002). Facultative siblicide in Serengeti hyenas, whereby the dominant sibling of a twin litter prevents the subordinate from obtaining sufficient milk by monopolising access to maternal teats, resulted in reduced growth rates and eventually the death of the subordinate from starvation (Hofer & East 1997). Growth rates of dominant cubs surviving in a litter in which siblicide occurred were the same as those of singletons, demonstrating that the dominant cub benefitted from the milk supply originally des-

tined for its sibling, and that mothers do not radically reduce milk delivery after litter reduction (Hofer & East 2008). Low prey abundance in the clan territories of the Serengeti hyena subpopulation is likely to reduce the rate at which cubs are nursed because their mothers commute, leading to increased sibling rivalry and decreased nutritional status, particularly in subordinate cubs of twin litters of low ranking females (Golla et al. 1999) which would most likely result in increased physiological ‘stress’. Therefore, nutritional ‘stress’ and physiological ‘stress’ are likely to be confounded. I would expect subordinate cubs of twin litters to be more susceptible to infection than dominant cubs or singletons. Cubs from twin litters also spend much time nursing together and alternate in the use of teats. I would expect that if one sibling is infected the chance to transmit pathogens to the other sibling is higher than in the case of singletons.

Factors influencing longevity

In spotted hyenas, offspring typically obtains a social status within the linear dominance hierarchy similar to and below that of their mother (Holekamp & Smale 1995; Smale et al. 1995; Engh et al. 2000; East et al. 2009). As social dominance provides fitness benefits, these benefits are therefore passed to offspring across generations (‘silver-spoon effect’; Hofer & East 2003). Because high-ranking females have priority of access to food in their territory, their offspring benefit from the silver-spoon effect through faster growth (Hofer & East 2003) and higher survival than offspring of subordinate females (Holekamp et al. 1996; Hofer & East 2003). Thus maternal social status is closely related to the nutritional status of offspring. Infections with a pathogen can be nutritionally demanding and deplete body resources. I would therefore expect that longevity may be reduced in cubs from low-ranking mothers, subordinate cubs in twin litters, and infected cubs.

To test which ecological parameters (and the likely physiological effects these have on cubs) explain infection patterns and to investigate the consequences of infection with non-virulent pathogens for individual longevity, I used demographic data collected during a 22-year long-term study on individually known spotted hyenas inhabiting the Serengeti National Park. Available data included life history parameters that may influence a young hyena’s susceptibility to acquire infections such as age, sex, social status, litter size, dominance status within a litter, or concurrent infection with helminths. I contributed the results of non-invasive screening (faecal samples) of several hundred individually known spotted hyenas for the presence of coronaviral and caliciviral RNA using RT-PCR methods (Chapter 3.1 and 3.2) and inspection for the presence of helminths.

3.3.2 METHODS

Assessment of viral and helminth infection

To determine prevalence of CoV and CV infection, faecal samples collected from known individuals were screened for the presence of viral RNA using reverse transcriptase PCR (RT-PCR) as described in detail in Chapters 3.1 and 3.2. As I observed different infection patterns in the Crater population, for instance no CoV infection (Chapter 3.1) and infection of only a few individuals with CV (18 individuals from 84 or 22 %, Chapter 3.2), I did not include infection data from Crater hyenas in this analysis. Faecal samples were checked for the presence of helminths (and/or helminth segments) immediately after sample collection in the field. Sample containing helminths (or segments) were scored as positive.

Statistics

To evaluate factors that influence viral infection in juvenile hyenas, I built a multinomial logistic regression model. I only included data from individuals of which the following information was available: (1) *Individual life history parameters*: Sex, dominance status of cubs within twin litters (subordinate/ dominant) with singletons treated as dominant cubs since dominant cubs have priority of access to milk (Hofer & East 2008), age (cubs/subadults), litter size (singleton/twins) and maternal social status. (2) *Indicators of individual nutritional and immune status*: Level of prey abundance inside the clan territory (low/medium/high) as defined by Hofer & East (1993a), presence or absence of helminth infection, interactions between sex and dominance status (subordinate male or female/dominant male or female) and between sex and helminth infection (infected male or female/non-infected male or female). In total, complete data parameters from 135 Serengeti juvenile hyenas (111 cubs, 24 subadults) screened for infection with CV and CV were available for analysis.

To assess which factors influence longevity of juvenile spotted hyenas, I conducted a survival analysis for right-censored data and applied the Cox proportional hazard model. I included the following parameters in the analysis: individual infection status (not infected, only infected with CoV, only infected with CV, co-infected with both viruses), maternal social status, dominance status within litter, sex, infection with helminths and level of prey abundance in the clan territory. I only considered data from cubs whose survival to two years of age and longer was monitored. I tested which of these factors increased the chance of mortality (cumulative hazard) and thus reduced longevity. Complete data on longevity as well as all other

factors included in the survival analysis were available from 114 juvenile hyenas. All statistical analyses were conducted with SYSTAT 12 (Systat Software Inc., Richmond, USA).

3.3.3 RESULTS

Factors influencing infection status

The probability of single and multiple infections was significantly influenced by several ecological and life history parameters as demonstrated by the results of the multinomial logistic regression model (model statistics: $G = 78.98$, d.f. = 33, $n = 135$; $p = 0.00001$). The effects of significant individual factors are summarized in Table 8. Significant overall effects in the model were observed in the age category, presence of helminths, litter size and prey abundance (Table 8). Factors that had an influence are briefly outlined below:

a) Significant factors influencing infection with coronavirus

Individuals who shed helminths in their faeces were less likely to be infected with CoV. Members of twin litters were more likely to be infected than singleton cubs. During times of medium prey abundance, the likelihood of being infected was lower than during times of either high or low prey abundance.

b) Significant factors influencing infection with calicivirus

Subordinate littermates were more likely to be infected with calicivirus than dominant cubs or singletons; cubs were more likely to be infected than subadults. I observed a significant interaction between sex and helminth infection in that males with helminth infection were less likely to be infected with calicivirus. Cubs of twin litters were more likely to be infected than singletons, and during times of medium prey abundance the likelihood of being infected was lower than during times of either high or low prey abundance.

c) Factors influencing co-infection with both viruses

Cubs were more often co-infected with both viruses than subadults. Individuals that shed helminths were less likely to be co-infected than individuals that did not shed helminths. The likelihood of co-infection increased with maternal social rank and during times of medium prey abundance.

Table 8: Factors influencing infection with either CoV or CV, and those that influenced co-infection with both viruses. In bold: significant p-values.

Factor		CoV-infected	CV-fected	in-Co-Infected	Overall effect
Dominance status	subordinate siblings	0.311	↑ 0.031	0.133	0.135
Age category	cubs < 12 months of age	0.198	↑ 0.0058	↑ 0.0045	0.0077
Helminth infection	infected individuals	↓ <0.0001	0.522	↓ <0.0001	<0.0001
Sex * helminths	infected males	n.a.	↓ 0.024	n.a.	0.163
Number cubs	-	0.892	0.199	0.262	0.153
Litter size	twin litters	↑ 0.0012	↑ 0.002	0.698	0.001
Social status	Offspring of low-ranking mothers	0.788	0.149	↑ 0.035	0.108
Level of prey abundance	juveniles during times of medium prey abundance	↓ 0.00018	↓ 0.0006	↓ 0.0059	0.001

n.a.: not applicable

↑: increased likelihood of infection

↓: reduced likelihood of infection

Factors influencing longevity of juveniles

The survival analysis as implemented with the Cox proportional hazard model demonstrated that the chance of mortality (cumulative hazard) was significantly influenced by social factors and infection category ($G = 12.50$; d.f. = 5; $n = 114$; $p = 0.03$). Survival analysis revealed that infection status had a significant influence on the longevity of juveniles (see Table 9). Individuals that were infected with CoV had a significantly higher cumulative hazard, and thus a reduced longevity, than individuals infected with CV, individuals co-infected with both viruses, or not infected individuals (Fig. 10).

Other parameters that had a significant effect on longevity included maternal social status, whereby offspring of lower-ranking mothers had a reduced longevity, and dominance status within litters in which subordinates showed reduced longevity. Sex, presence or absence of helminth infection and levels of prey availability did not influence longevity of individuals (Table 9).

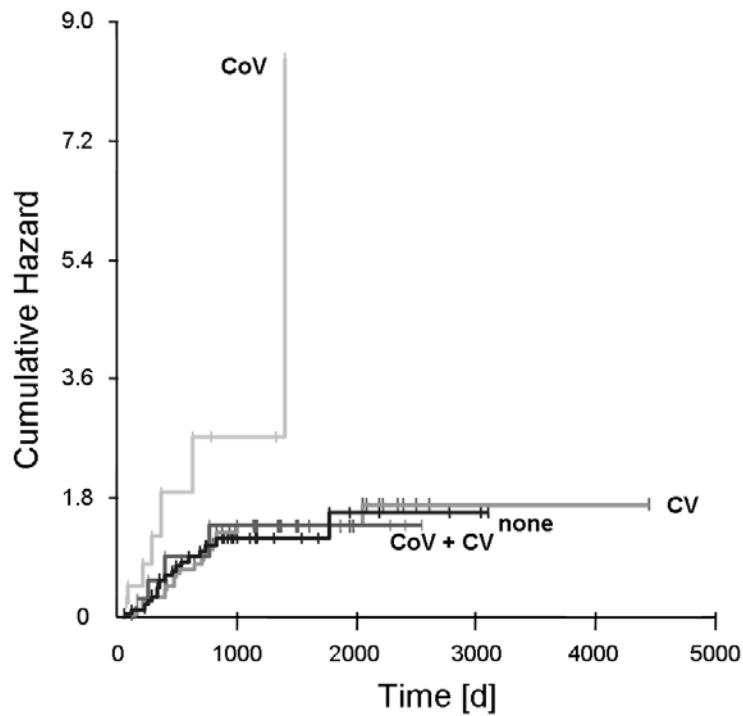


Figure 10: The likelihood of mortality (cumulative hazard) as a function of individual age (days) of cubs with differing infection status at the time of sampling: no infection ('none', black), infection with CoV ('CoV', light grey), infection with CV ('CV', grey) or co-infection with both viruses ('CoV+CV'; dark grey). The cumulative hazard was calculated using Kaplan-Meier survivorship probabilities based on data on the longevity of all cubs for which all data were available.

Table 9: Factors and their effect on longevity of juvenile hyenas tested in a Cox proportional hazard model for survival analysis. In bold: significant p- values.

Factor	Effect	p-value
Infection status (not infected, corona-only, calici-only, co-infected)	Longevity differs between categories of infection status (worst effect: corona infection)	0.044
Social status of mother	High-ranking offspring live longer	0.008
Dominance status within litter	Dominants live longer	0.048
Sex	-	0.643
Infection with helminths	-	0.268
Period of high prey abundance	-	0.283

3.3.4 DISCUSSION

The results of this Chapter provide evidence that key life history and ecological parameters linked to physiological effects significantly influenced infection status of juvenile spotted hyenas. The most important factors to reduce the likelihood of viral infection included a concurrent infection with helminths and a medium level of prey abundance in the territory. Cubs and members of twin litters were more likely to be infected than subadults and singletons.

Longevity was decreased in spotted hyenas infected as juveniles with CoV and in subordinate cubs from twin litters and declined as maternal social status declined.

Concurrent helminth infection

In contrast to my expectation, concurrent infection with helminths reduced - not increased - the likelihood of co-infection with CoV and CV. The likelihood of infection with CoV was reduced in cubs of both sexes with concurrent helminth infection; the same was true for cubs of both sexes co-infected with both viruses. The presence of helminths decreased the chance of acquiring CV infection only in male cubs.

A possible explanation for the effect of helminth infection on the reduced prevalence of infection with these two enteric viruses could be an induction of a local immune response of the host in the mucosal epithelium in the intestines. It is known that the mucosal surface of the gastrointestinal tract is replete with antigen reactive lymphoid tissues, the so called gut-associated lymphoid tissue (GALT) which includes B cells, T lymphocytes, plasma cells and other cellular elements involved in the induction and maintenance of immune responses (e.g. MacDonald 2003). Helminth infection in humans induces the secretion of IgEs and IgGs and several interleukins such as IL-4 and IL-13 that play a role in preventing the development of autoimmune responses (Carvalho et al. 2006). Helminth infection stimulates B cells responsible for the secretion of IgAs. It is suggested that secretory (s)IgAs and sIgGs in the enteric tract may play a role in local protection against rotavirus infection in pigs (Yuan et al. 1996) and against CoV infection in dogs (Decaro et al. 2004). It therefore might be possible that juvenile hyenas with helminth infections may secrete sufficient antibodies to prevent the attachment of coronaviral particles to the surface of epithelial cells, thereby decreasing the chance of CoV infection. However, the role that sIgs play in protection against enteric viruses is still uncertain and needs further investigations.

Age

Age often plays a role in infection. As already outlined in Chapters 3.1 and 3.2, CoV and CV infection in spotted hyenas is more likely to occur in young individuals than in adults. Female spotted hyenas typically only nurse their own offspring but young are reared together in a communal den for approximately 12 months (Hofer & East 1993c). Thus, numerous cubs shelter together in underground burrows during the day, thereby increasing the chance of viral transmission from infected to susceptible cubs (Höner et al. 2008). However, the number of cubs occupying the communal den did not influence infection pattern. This suggests, similar to the transmission of other viruses such as rabies (Anderson et al. 1981), a non-linear effect

such as a threshold contact rate beyond which an increase in contact rate or group size has little effect on transmission dynamics. In that case, ‘group sizes’ of cubs at Serengeti communal dens are sufficiently high to exceed the threshold contact rate.

Young cubs use communal latrines in the vicinity of the den and regularly sniff the anogenital area of other cubs during ritualised greeting ceremonies. Both behaviours provide considerable potential for pathogen transmission (East et al. 2004). Unlike older animals, spotted hyena cubs younger than 12 months of age have a range restricted to the vicinity of the communal den, and thus the viruses they are likely to be infected with are most likely to be transmitted to them from other cubs at the den or by other clan members that visit the den. The probability of viral transmission from older clan members to den-bound cubs will be modulated by the level of herd immunity to any particular virus in older animals.

Litter size, dominance status and maternal social status

As predicted, cubs raised in twin litters were generally more likely to be infected with either CoV or CV whereby subordinate littermates additionally had an increased chance of having CV infection (Table 8) and their longevity was reduced (Table 9). The early establishment of dominance relationships between members of twin litters strongly influences the access of dominant and subordinate littermates to maternal milk (Hofer & East 1997). Provided mothers deliver sufficient milk, cub growth rates between members of twin litters did not substantially differ and siblicide did not occur (Golla et al. 1999; Wachter et al. 2002) but when maternal attendance at the den declined, the asymmetry in growth rates between the dominant and subordinate littermate increased (Hofer & East 2008), rates of aggression rose (Golla et al. 1999) and the chance of facultative siblicide increased (Hofer & East 2008). Elevated levels of aggressive conflict between littermates are likely to result in increased physiological ‘stress’ in terms of increased glucocorticoid concentrations (Hofer & East 1998; Goymann et al. 2001). It is therefore possible that subordinate littermates in Serengeti twin litters suffered both nutritional and physiological ‘stress’ during periods when their mothers traveled long distances to forage and were repeatedly absent for several days (Hofer & East 1993b, 2008). The combination of both stressors is likely to reduce immunocompetence in twin litters, particularly in subordinate cubs, compared to singletons. This may explain the increased likelihood of subordinate cubs being infected with CV and the generally reduced longevity of subordinate cubs.

Results obtained from the survival analysis suggest that longevity increased with maternal social status. Quality of maternal care depends on maternal social status because high-ranking

females have priority of access to food within a clan territory (Hofer & East 2003). In contrast, low-ranking females go on long distance commuting trips to forage on migratory herds outside the clan territory. As a consequence, offspring of low-ranking females grew more slowly and had a reduced chance of survival (Hofer & East 2003). Consistent with these results, my survivorship analysis showed that longevity of spotted hyenas with known infection status during their juvenile stage improves with maternal social status.

Prey availability

The likelihood of acquiring single infections or co-infection with both enteric viruses was higher during times of low or high prey abundance than during times of medium prey abundance.

Why was susceptibility of juvenile hyenas high during times of low prey availability? In the Serengeti National Park, the abundance of resident herbivores is low and most herbivores undertake annual long-distance migrations. The larger the distance between the communal den and the area in which females feed, the longer the interval between successive deliveries of milk to their offspring (Hofer & East 1993c). As shown by Hofer & East (2003), the lower the maternal input the slower the growth rate of dependent cubs and the lower the survival to the age of independence at 2 years of age. Restricted maternal input during times of low prey abundance could result in severe nutritional stress that might compromise immune responses to pathogens, thus increasing the susceptibility of young hyenas to infection. A decreased allocation of nutrients to immune responses in juveniles is probably a consequence of a life-history trade-off between the substantial cost of developing immune responses and the high cost of growth (Gulland 1992; Sheldon & Verhulst 1996; Nelson 2004).

In contrast to expectation, the chance of infection was also higher during times of high prey abundance than during times of medium prey abundance. Prevalence of infection in a host population is likely to be elevated when high contact rates between infected and susceptible individuals permit efficient pathogen transmission. Therefore, when host abundance is high, prevalence of infection might be elevated (Hudson et al. 2002) unless it is prevented by substantial levels of herd immunity. During periods of high prey abundance, most members of a clan regularly gather around the communal den, the social centre of a clan where most social interactions take place (East & Hofer 1991). When high concentrations of prey are in a clan territory, lactating females nurse their cubs daily (Hofer & East 1993c). During periods of high prey abundance, the high number of contact partners (Hofer & East 1993b; East & Hofer 2001) and the potentially high number of friendly or aggressive social interactions with other

clan members are likely to increase the chance of pathogen transmission (see also East et al. 2001).

Taking these two explanations for the increased likelihood of infection during low and high prey availability into account, it might be possible, that an ‘optimal balanced’ interplay between nutritional status and contact rates during times of medium prey availability may keep the likelihood of infection low.

In summary my results suggest that levels of infection among spotted hyena cubs are influenced by the interplay of several factors including nutritional state, physiological ‘stress’, contact rates, maternal social status as well as interactions between micro-parasites and macro-parasites. Both enteric viruses considered here, CoV and CV, produced no obvious or confirmed clinical symptoms, yet CoV infection during the juvenile stage significantly reduced longevity, suggesting that a life history perspective on host demographics will improve our understanding of the individual consequences as well as long-term evolutionary effects of such subclinical infections.

3.4 Persistence of coronavirus within a metapopulation of spotted hyenas

3.4.1 INTRODUCTION

The economic costs of infectious pathogens and the threat they pose to human health, agricultural production and the preservation of global biodiversity (Hess 1996; Pederson et al. 2007; Jones et al. 2007; Smith et al. 2009) has engendered considerable interest in factors that influence host-pathogen dynamics within individual hosts (Paterson et al. 1998; Fenton et al. 2006; Graham et al. 2007; Beldomenico et al. 2008) and how these and other factors modulate the dynamics of pathogen infection at the host population level (Anderson & May 1979a,b, see also 1991; Diekmann & Heesterbeek 2000). For simplicity, classical infectious disease models assume complete mixing of host populations (whereby each infectious individual is as likely to infect each susceptible individual in the population) which is unlikely to be valid for social, group-living mammalian hosts, including humans (Read & Keeling 2003; Davidson et al. 2008; Jesse et al. 2008). More recent models consider spatial heterogeneity or factors such as age-dependent transmission rates in various ways. Host interactions can be modelled to occur across a continuum between completely local interactions at one extreme and completely global at the other extreme (Boots & Sasaki 1999; Keeling 1999; Read & Keeling 2003). The metapopulation concept (Gilpin & Hanski 1991) as applied to disease modelling (Grenfell & Harwood 1997) can consider heterogeneity by modelling pathogen dynamics separately for each subpopulation (Hess 1996; Park et al. 2002; Jesse et al. 2008) or by considering whole subpopulations as infectious, susceptible or recovered (Hess 1996; Gog et al. 2002). Furthermore, infection in one subpopulation can be considered to lead to infection in surrounding subpopulations without specifying how transmission occurs (Park et al. 2002; Hagenaars et al. 2004) or infection can spread when an infectious host from one subpopulation is mechanistically moved to another (Keeling & Rohani 2002; Cross et al. 2005).

Pathogens are expected to adapt to available routes of transmission from infectious to susceptible individuals. When transmission occurs within local networks composed of small clusters of highly connected individuals in which each infectious individual has many susceptible neighbours, progeny of a viral strain are likely to be in direct competition for available hosts. This competition would be expected to drive selection for an increase in transmission rate. However, selection for increasing transmission rates in pathogens that utilise local clusters has the disadvantage that infection would spread rapidly through all available susceptibles within a cluster, thereby exhausting the host resource (host ‘burn out’) and leading to the local extinction of the strain (Rand et al. 1995; Read & Keeling 2003). The probability of host ‘burn

out' depends on the appearance of new susceptibles (typically through births) in the host population. Immunity to re-infection among hosts recovered from an infection would be expected to create 'barriers' of immune individuals that would prevent pathogen transmission between clusters of susceptibles. These barriers should wane over time as recovered animals die or immunity declines (Read & Keeling 2003).

More complex transmission modes may have to be considered, since several host traits may play an important role in driving transmission dynamics. Seasonality of breeding for example can boost recurrent infections through the introduction of new susceptibles in a group (Lloyd-Smith et al. 2005; Conlan & Grenfell 2007), social aggregations during the reproduction season can increase transmission dynamics and thus spread of disease (Hosseini et al. 2004) and parameters such as social group size, recruitment rate and movements can affect pathogen persistence (Cross et al. 2005, 2007).

Relatively few empirical studies have investigated the persistence and spread of an infectious virus within and between subpopulations of the metapopulation of a large mammal (Grenfell & Harwood 1997; de Castro & Bolker 2005). Infectious diseases that are endemically transmitted in their host population may experience extinction if the chain of transmission terminates or is interrupted. Pathogens inducing life-long, full immunity to re-infection in populations that only contain few new susceptible hosts tend to become extinct (Keeling & Grenfell 1998; Grenfell & Dobson 1995). Furthermore, the ability of a virus to persist in a host population depends on its basic reproductive rate R_0 . R_0 is a key concept in epidemiology and defined as the expected number of secondary infections arising from a single host individual during his or her infectious period in a fully susceptible population. If $R_0 < 1$, each infectious individual produces on average less than one new infected individual and the virus is likely to be cleared from the population. If $R_0 > 1$, the pathogen is able to invade and persist in a susceptible population. During recent years, theoretical work on R_0 was extended for investigating complex models such as age-class dependent transmission patterns (Anderson & May 1991) or spatially structured populations (e.g. Lloyd & May 1996; Keeling 1999).

In this Chapter I investigate the transmission and persistence of an infectious virus (CoV) in a spotted hyena metapopulation in Tanzania. CoV is transmitted by viral inhalation or ingestion during close social contact between a susceptible and an infectious individual, or through contact by a susceptible individual with contagious faeces which may occur when spotted hyenas defecate in communal latrines (East et al. 2004). The spotted hyena metapopulation included one subpopulation in the Serengeti National Park (referred to as Serengeti subpopulation) and another in the adjacent Ngorongoro Crater (referred to as the Crater subpopulation; see Fig. 1,

Chapter 2). The Crater caldera wall formed a physical barrier that appears to have prevented female dispersal between these two subpopulations as documented by an mt-DNA haplotype in the Crater distinct from those in the Serengeti subpopulation (Albert et al. 2000). In contrast, the presence of a few immigrant males in the Crater with the Serengeti mt-DNA haplotypes demonstrates that dispersing males relatively seldom (on average one male per year, Höner et al. 2005) traverse the Crater wall and may immigrate into local clans (Albert et al. 2000).

Spotted hyenas in both the Serengeti and the Crater share the same social structure (Hofer & East 1993a,b; Höner et al. 2005; see Chapter 2) but differ in the extent of their ranging behaviour (Hofer & East 1993a; Höner et al. 2007, Chapter 2). Ranging behaviour, social status, sex and age influence the nature and frequency of interactions between clan members (East et al. 1993, 2001; East & Hofer 2001; Höner et al. 2007). The Serengeti subpopulation is large and stable (approximately 5300 individuals, Hofer & East 1995) while the Crater harbours a smaller but growing population (approximately 400 individuals, Höner et al. 2005). All adult females reproduce and cubs are born throughout the year in both the Crater and Serengeti (Höner et al. 2005; Hofer & East 2008). For at least the first 12 months of life, cubs are stationed at a communal den in the clan's territory (Hofer & East 1993c, 2003; Wachter et al. 2002) and thus have high daily contact rates amongst themselves at and in communal dens (Höner et al. 2006) and frequent interactions with other clan members (East et al. 1993, 2001) but not with individuals from other groups (Hofer & East 1993b). Den-based cubs do not venture far from the vicinity of the communal den, thus their range is much smaller and restricted than that of older animals (Hofer & East 1993c; Höner et al. 2005).

Here I considered the metapopulation to consist of animals susceptible to infection, infectious individuals that transmitted coronavirus to susceptibles, and animals that had recovered from infection. I assumed animals were susceptible to infection from birth until infected, infectious individuals were those identified by reverse transcriptase (RT)-PCR methods with coronaviral RNA in their faeces (see Chapter 3.1) and recovered animals were those that no longer had coronaviral RNA in their faeces after initial infection and those with significant coronaviral antibody titres indicating previous exposure (East et al. 2004). I calculated R_0 for several age categories from birth until adulthood by considering age-specific mortality rates and the reduction of the proportion of susceptibles between successive age categories. I also determined an estimate of the infectious period by using data obtained from a few individuals from which an initial non-infectious sample, a subsequent infectious sample, and one or several subsequent non-infectious samples were collected. I investigated differences in the availability of

susceptible individuals in both subpopulations by determining the frequency and duration of periods during which susceptible cubs were present or absent in each population.

3.4.2 METHODS

Study sites and study animals

Data were obtained from three spotted hyena clans in the Serengeti National Park for approximately 22 years (May 1987- March 2009), 20 years (November 1989 – March 2009) and 19 years (August 1990 – March 2009) and all eight resident clans on the Crater floor (an area of 250 km²) for approximately 12 years (April 1996/ February 1997 – December 2008). Members of all study clans were individually known (Hofer & East 1993a, b; Höner et al. 2005). Further details on both study subpopulations are outlined in Chapter 2.

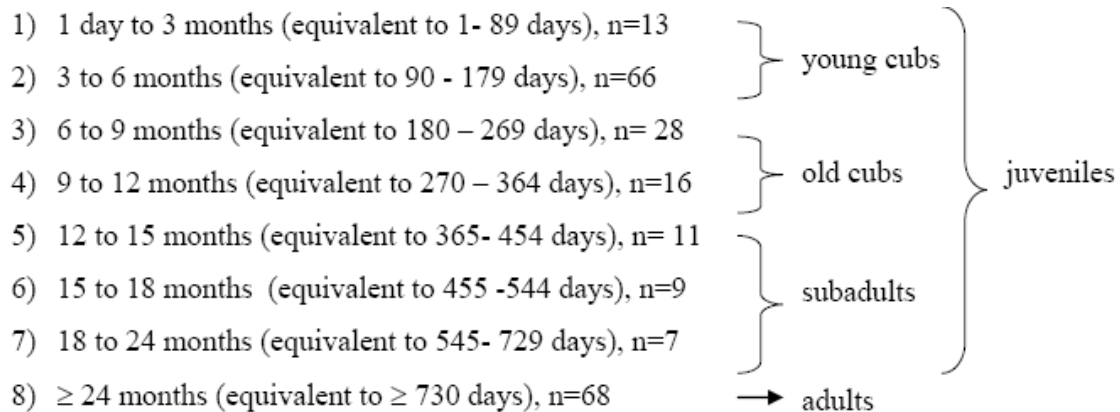
Assessment of viral infection

To determine the prevalence of CoV infection, I screened faecal samples collected from known individuals for the presence of coronaviral RNA using reverse transcriptase PCR (RT-PCR) as described in detail in Chapter 3.1. I screened a total of 350 faecal samples from 218 Serengeti individuals (123 cubs, 27 subadults, 68 adults) collected between 1995 and 2007 and 86 samples from 82 Crater individuals (42 cubs, 10 subadults, 30 adults) collected between 1997 and 2005.

To investigate exposure of hyenas to previous infection, colleagues (Prof. Karin Möstl and Dr. Viviane Benetka; VU Wien, Austria) screened serum samples from 87 Serengeti individuals (19 cubs, 11 subadults, 57 adults) collected between 1988 and 2009 and 9 samples from Crater individuals (2 cubs, 1 subadult, 6 adults) between 2002 and 2008 for the presence of antibodies. Briefly, serum samples were tested for antibodies against coronaviruses of the antigenic group 1 by indirect immunofluorescence assay (Moestl 1983) using Crandell feline kidney cells (CRFK) grown in microtitre plates. All sera were tested in two-fold serial dilutions (1:10 to 1:320). The conjugate used was a Fluorescein-conjugated AffiniPure Goat Anti-Cat IgG (Jackson Immuno Research Lab. Inc., West Grove, A, USA). The highest dilution showing a clear cytoplasmic fluorescence was recorded positive. For confirmation, a serum neutralisation assay was performed in CRFK cells inoculated with 100 TCID₅₀ (tissue culture infectious dose 50%)/0.1 ml of TGEV (strain Purdue), FCoV and CCoV in microtiter plates. The sera were used in two-fold serial dilutions (1:4 to 1:64) and the titre was recorded as the 50% inhibition dilution.

Determination of the proportion of susceptible and immune animals

I calculated proportions of infected and not infected individuals by the number of positive and negative samples detected in different age categories. I subdivided juvenile and adult hyenas in eight age categories between birth and 24 months of age as follows:



Cubs are hyenas between day one after parturition and 12 months of age and stationed at the communal den (Hofer & East 1993c; Wachter et al. 2002), thus their range is much smaller and restricted than that of older animals. Cubs were further subdivided into young cubs (up to 6 months of age) and old cubs (between 6 and 12 months of age) because young cubs are entirely dependent on maternal milk (Hofer & East 1993c) and rarely undertake excursions from the den whereas older cubs are more likely to undertake excursions and consume or scavenge meat in increasing proportions. The periods for both younger and older cubs were subdivided to acknowledge the increasing confidence of cubs moving about and participating in social interactions with or without the presence of their mother. Subadults are hyenas between 12 and 24 months of age. Subadults were divided into younger subadults (up to 15 and 18 months) because weaning usually takes place at around that time in the Serengeti and subadults still follow closely their mother inside the clan territory and on foraging excursions. Subadults older than 18 months typically move more independantly and may stay for extended periods away from the communal den (unpublished observations). The term juvenile hyenas encompassed both cubs and subadults. All hyenas older than 24 months of age were regarded as adults.

I assumed that infected individuals developed life-time resistance to re-infection based on a previously determined high sero-prevalence (70-90%) amongst adults in the Serengeti population (East et al. 2004), a lack of evidence of re-infection in eight individuals screened after clearance of an initial infection and the low proportion of RT-PCR results from faeces from

adult hyenas (1.5% of 68 Serengeti adults; this study). Once an animal was detected to be RT-PCR positive, it was assumed to be immune and thus excluded from the susceptible part of the population. I calculated the proportion of susceptible hyenas (p_{sus}) for each age category i by using the following equation in which the terms A and B represented the proportion of cubs not infected in the current age category i . The term C is equivalent to the sum of the proportion of infected (p_{inf}) cubs plus the proportion of ‘immunised’ cubs of the previous age category $i-1$. B multiplied by C represents the proportion of ‘immunised’ hyenas in the current age category i :

$$p_{\text{sus } i} = 1 - \underbrace{p_{\text{inf } i}}_A - \underbrace{[(1 - p_{\text{inf } i}) * (1 - p_{\text{inf } i-1})]}_B$$

$\underbrace{\hspace{10em}}_C$

Basic reproductive rate R_0

The basic reproductive rate R_0 is the average number of secondary cases of infection which one infected individual produces in a population only consisting of individuals susceptible to infection. CoV infection will persist within a subpopulation when $R_0 > 1$. I calculated R_0 for mortality schedule “II” (Anderson & May 1991) which expects a newborn individual’s probability to survive to age a declines exponentially with a for a stable population. I used the life expectancy L of juvenile hyenas to reach 730 days of age in the Serengeti and the average age A at infection for each age class ($R_0 = L/A$; see Anderson & May 1985). Average age at infection was the sum of infectives of each age category 1-8 divided by the sum of the proportion positives of the respective age category.

Infectious period and re-infections

To obtain an estimate of the duration of viraemia for CoV being shed by infected spotted hyenas I used data for those individuals ($n = 3$) for which I had obtained an initial non-infected sample, a subsequent infected sample and at least one further sample collected after the infection was cleared. To investigate whether hyenas might be re-infected after initial infection I used data from five additional individuals where one or more successive samples were collected after the first positive tested sample.

Presence of susceptible individuals in individual clans

A critical factor to viral persistence in spotted hyena populations is the presence of susceptible, naïve cubs at communal dens. I therefore determined periods when young cubs less than six months of age were absent (termed hereafter ‘absence periods’) from Serengeti and Crater

communal dens. These were calculated as the duration of the interval between two successive periods in which cubs of this age were present (termed hereafter ‘presence periods’). Presence periods are periods during which one or more cubs were present at the den and the duration of periods is given in days. As infection in subadult animals might contribute to viral persistence during short absence periods I used two minimum durations of periods when the absence of susceptible cubs was expected to affect virus transmission: firstly an absence of susceptible cubs for at least 21 day based on the minimum average period of viral shedding in dogs (9 days) and cats (~ 4 weeks) and secondly, an absence of susceptible cubs of 68 days, the minimum estimated excretion period of spotted hyenas as determined in this study (see results). Absence periods shorter than these minimum durations were excluded from the respective statistical analyses.

Statistics

I calculated log-likelihood ratio chi square tests to investigate associations between age and antibody prevalence and viral excretion across years. To assess the distribution of absence periods t for both the Crater and Serengeti data I calculated the Kaplan-Meier survivorship function $F(t)$ that incorporated right-censored data. I looked at differences in the duration of absence periods between both subpopulations using a non-parametric log-rank-test (Tarone & Ware 1977). Using the same analysis, I looked at differences in the duration of presence periods with young cubs. To look at differences in the proportion of absence periods in the Crater and Serengeti I performed Mann-Whitney U-Tests, taking each clan as the source of one data point each. I calculated the proportion of absence periods by dividing the number of absence periods by the total number of periods throughout the whole observation time. Median values and range are given for the duration of absence and presence periods. To look at differences in the number of cubs present in both subpopulations I calculated a Mann-Whitney U Test taking as source of one data point the median value of cubs present in each clan from each subpopulation across the whole observation.

3.4.3 RESULTS

I screened 350 faecal samples from 218 known animals in the Serengeti subpopulation and 86 faecal samples from 82 known animals in the Crater subpopulation (Höner et al. 2005) for the presence of coronaviral RNA (see Chapter 3.1). In the Serengeti, 31 of 218 individuals were infected in different years between 1995 and 2007 (Table 10) whereas none of 82 individuals in the Crater were infected between 1997 and 2006. As already shown in Chapter 3.1, the

prevalence of infectious individuals in the Serengeti was significantly higher in cubs than hyenas older than 12 months of age.

Table 10: CoV prevalence across years in cubs and hyenas older than 12 months of age (ad). Absolute numbers of positively tested animals and total number of tested samples are given. Percentages are given below absolute numbers.

Age	1995	1996	1997	1998	2001	2002	2003	2004	2005	2006	2007	Total
cub	1/1 100	1/1 100	1/1 100	-	1/3 33	1/6 17	5/17 29	3/16 19	1/10 10	1/34 3	11/34 32	26/123 21
ad	-	-	1/6 17	1/2 50	1/11 9	0/3 0	1/4 25	0/1 0	0/21 0	0/20 0	1/27 4	5/95 5

Viral excretion of cubs varied across years (log-likelihood ratio Chi square, $\chi^2 = 13.3$; d.f. = 5; $n=117$; $p = 0.02$; statistical analysis only includes years with $n > 5$), with the highest levels of infection being observed in 2003 and 2007 and particularly low infections between 2001 and 2002 as well as 2004 until 2006 (Table 10). Viral excretion of adults did not vary across years (log-likelihood ratio Chi square, $\chi^2 = 7.7$; d.f. = 4; $n = 83$; $p = 0.1$; statistical analysis only includes years with $n > 5$).

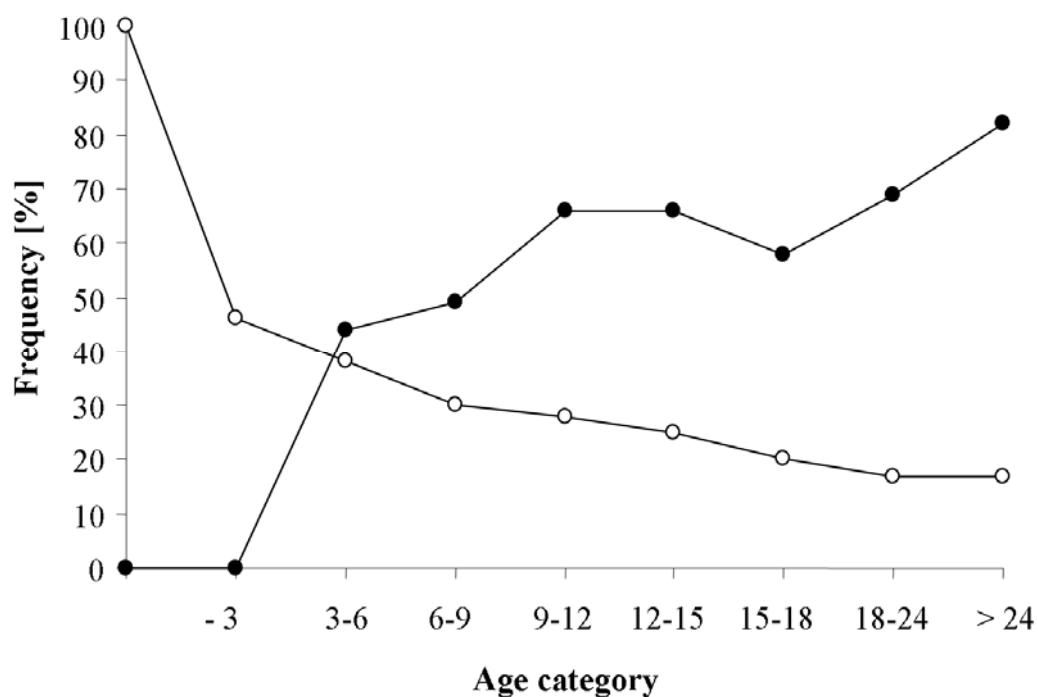


Figure 11: The effect of age on the proportion of spotted hyenas susceptible to CoV infection (○) and the proportion previously exposed to the virus and likely to be immune to re-infection (●). Age categories are given in months.

The proportion of susceptibles decreased rapidly with age as an increasing number of animals were exposed to and recovered from infection (Fig. 11), so that herd immunity in the adult

Serengeti subpopulation was high (81.9 % immune, Table 11). Most infections were detected in young cubs up to three months of age (53.8 % infected) and proportions of susceptible hyenas was highest in the first two age categories (46.2 % and 37.8 % susceptible, Table 11).

Table 11: Proportions of infected, immune and susceptible hyenas in different age categories.

Age category [months]	0-3	3-6	6-9	9-12	12-15	15-18	18-24	≥ 24
Infected [%]	53.8	18.2	21.4	6.3	9.1	22.2	14.2	1.5
Immune [%]	0.0	44.1	48.9	65.9	65.6	58.1	68.9	81.9
Susceptible [%]	46.2	37.8	29.7	27.8	25.3	19.7	16.9	16.6

Long-term serological data of 19 cubs and 68 individuals ≥ 12 months of age (East et al. 2004; this study) demonstrates that Serengeti hyena cubs were exposed to CoV between 1988 and 2009 (Table 12) and suggests that infection was endemic in the population. There was a significant difference in seropositivity in that cubs were less often seropositive (53% positive) than subadults and adults (82% positive; log-likelihood ratio $\chi^2=6.5$; d.f. = 1; n = 87; p = 0.01). Unfortunately, sample sizes in most years were too low to statistically analyse differences in seroprevalence across years. Only limited serological data from the Crater population was available with three of five (60%) adults seropositive and the two cubs and the one subadult tested negative for exposure to CoV.

Table 12: Seroprevalence of Serengeti hyena cubs and hyenas older than 12 months of age (ad) across years. Absolute number of positively tested animals and total number of tested samples are given. Percentages given below absolute values.

	1988	1989	1991	1994	1995	1996	1997	1998	1999	2000	2001	2002	2003	2005	2006	2007	2009	N
cub	1/1 100	-	-	-	2/3 67	1/1 100	3/5 67	-	2/2 100	-	0/2 0	0/1 0	1/4 25	-	-	-	-	10/19 53
ad	18/27 67	3/3 100	1/1 100	5/5 100	9/9 100	5/5 100	5/5 100	1/1 100	2/2 100	2/2 100	1/1 100	-	1/1 100	1/1 100	1/1 100	1/3 33	1/1 100	56/68 82

Basic reproductive rate R_0

R_0 was highest when calculated for cubs up to three months of age (R_0 type II: 6.03) and cubs between three and six months of age (R_0 type II: 7.58) and decreased between successive age categories and reached its lowest value in adults (R_0 type II: 1.45; Table 13).

Table 13: Basic reproductive rate R_0 in different age categories. R_0 was calculated for mortality schedule type II.

Age category	R_0 type II
0-3 months	6.03
3-6 months	7.58
6-9 months	4.09
9-12 months	3.60
12-15 months	2.91
15-18 months	3.37
18-24 months	2.34
>24 months	1.45

Infectious period

Regrettably, the data are insufficient to accurately estimate the period of viremia. To obtain a rough maximum estimate I used data from juveniles of which we had collected a pre-infection, infection and post-infection sample and assumed viral excretion started on the day after the first negative sample pre-infection and ended on the day before the final negative sample post-infection (Table 14).

Table 14: Estimated maximum duration of the period during which an individual may be infectious. Given are the respective animal IDs, the collection dates of the negative samples pre-infection, of the positive sample and the first negative sample post-infection as well as the possible maximum period of viraemia.

Animal ID	Date tested negative pre-infection	Date tested positive	Date tested negative-post infection	Possible maximum period of viraemia [days]
M567	20070623	20070704	20070911	78
P506	20070820	20070902	20071111	81
P507	20070615	20070703	20070824	68

As all estimates were maximum estimates; I used the shortest estimate of 68 days as an approximation of the infectious period. None of five infectious animals tested positive for viral RNA in faeces when re-sampled after the date they were first tested positive (Table 15).

Table 15: Periods (days) between the first tested positive sample and subsequent tested negative samples.

Animal ID	Date tested positive	Date tested negative	Period [days]
I369	20030106	20030201	26
P509	20070920	20071201	72
M566	20070704	20070924	82
M573	20070628	20070901	65
		20070906	70
		20071010	104
I357	20030306	20030714	130

Persistence of the presence of susceptibles

As CoV was predominantly transferred among den-bound cubs (Table 11), one key factor influencing persistence of infection at both the group and subpopulation level was the presence of susceptible juveniles. The age category containing the highest proportion of susceptibles (37.8 - 46.2 %) was young cubs up to 6 months of age (Table 11). During 22 years of monitoring of three clans in the Serengeti subpopulation, there were only 5 periods (median duration 36 days, range 5-67 days) when cubs less than 6 months of age were absent (see Fig. 12). During 12 years of monitoring of all eight clans from the Crater subpopulation, there were in total 54 periods (median duration of 66.5 days, range 2 - 417 days) when cubs less than 6 months of age were absent (see Fig. 13; only exemplary data from three clans are shown). The number of cubs present in each subpopulation was significantly higher in the Serengeti subpopulation than in the Crater subpopulation (Mann-Whitney U-test, $U = 1.0$, $n_{\text{Serengeti}} = 3$, $n_{\text{Crater}} = 8$; $p = 0.014$). Considering all absence periods of potential hosts, absence periods lasted significantly longer in the Crater than in the Serengeti subpopulation (Tarone-Ware, $\chi^2 = 8.34$; d.f. = 1; $n = 59$; $p < 0.004$), and the occurrence of such periods were more likely in the Crater than in the Serengeti (Mann-Whitney U-test, $U = 24.0$; $n_{\text{Serengeti}} = 3$, $n_{\text{Crater}} = 8$; $p = 0.014$). If spotted hyenas are only infected for approximately 21 days (average infectious period typically for infected domestic dogs and domestic cats), there were only 3 periods without cubs in the Serengeti subpopulation (median duration 48 days, range 36-67 days) in contrast to 37 periods (median duration 100 days, range 24-417 days,) in the Crater subpopulation. Absence periods that exceeded my estimate of the duration of viraemia of 68 days were not recorded in the Serengeti subpopulation but 27 such absence periods occurred in the Crater (median duration 137 days, range 72-417 days).

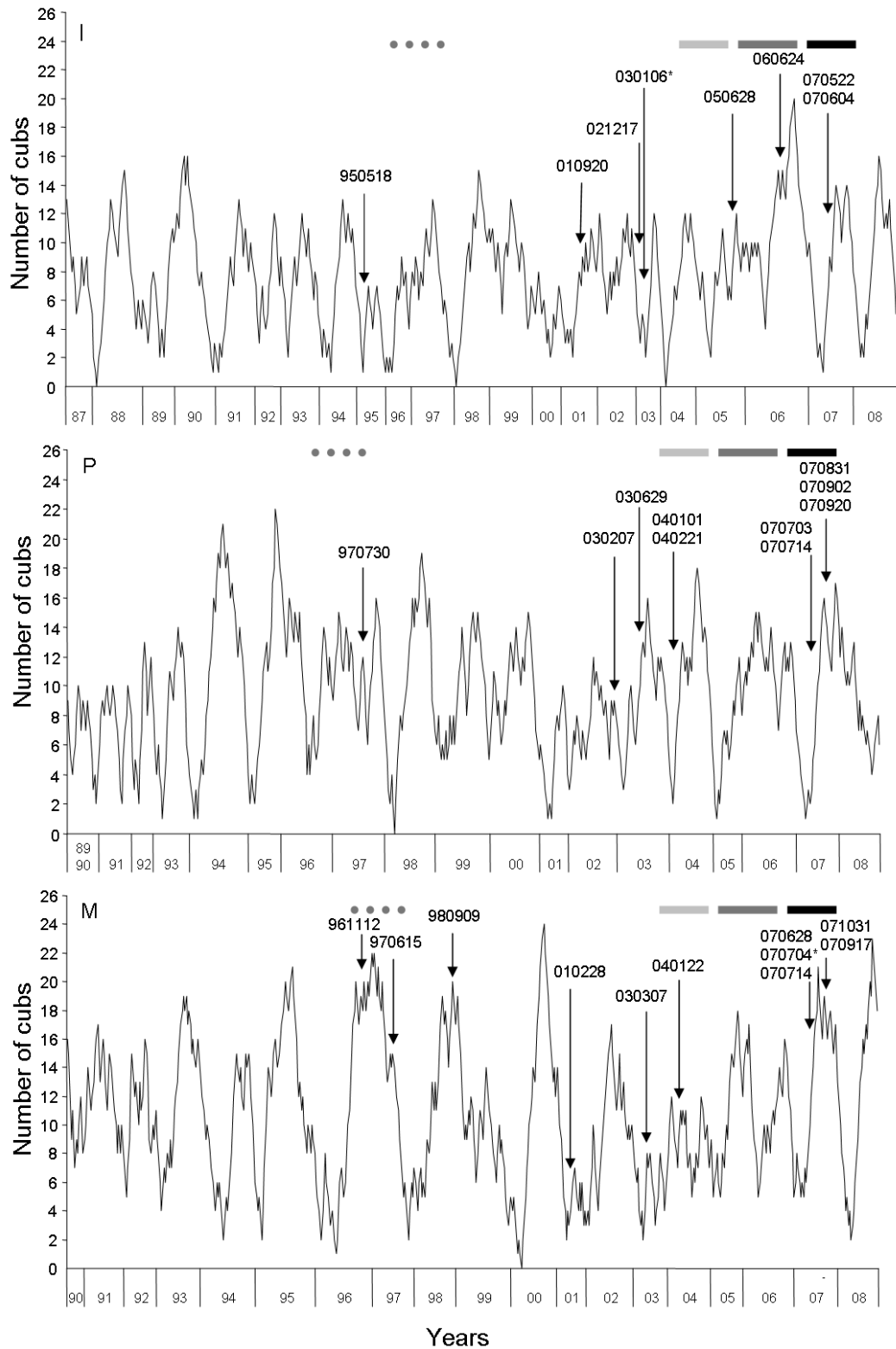


Figure 12: Absence and presence periods with increasing and decreasing numbers of cubs present in each clan across the whole observation time. Data of the three study clans in the SNP are shown. Exact duration of each period is not considered in this figure. Additional information: bars indicate when different CoV variants circulated in the Serengeti hyena subpopulation: dotted grey bar: FCoV-like variant in 1997; light grey bar: FCoV-like variant and CCoV-like variant in 2004; dark grey bar: CCoV-like variant in 2006; black bar: CCoV-like variant in 2007. Collection dates of the 31 positive tested faecal samples from individual hyenas (see Chapter 3.1) are given and indicated by arrows. * two positive samples were obtained on the same day

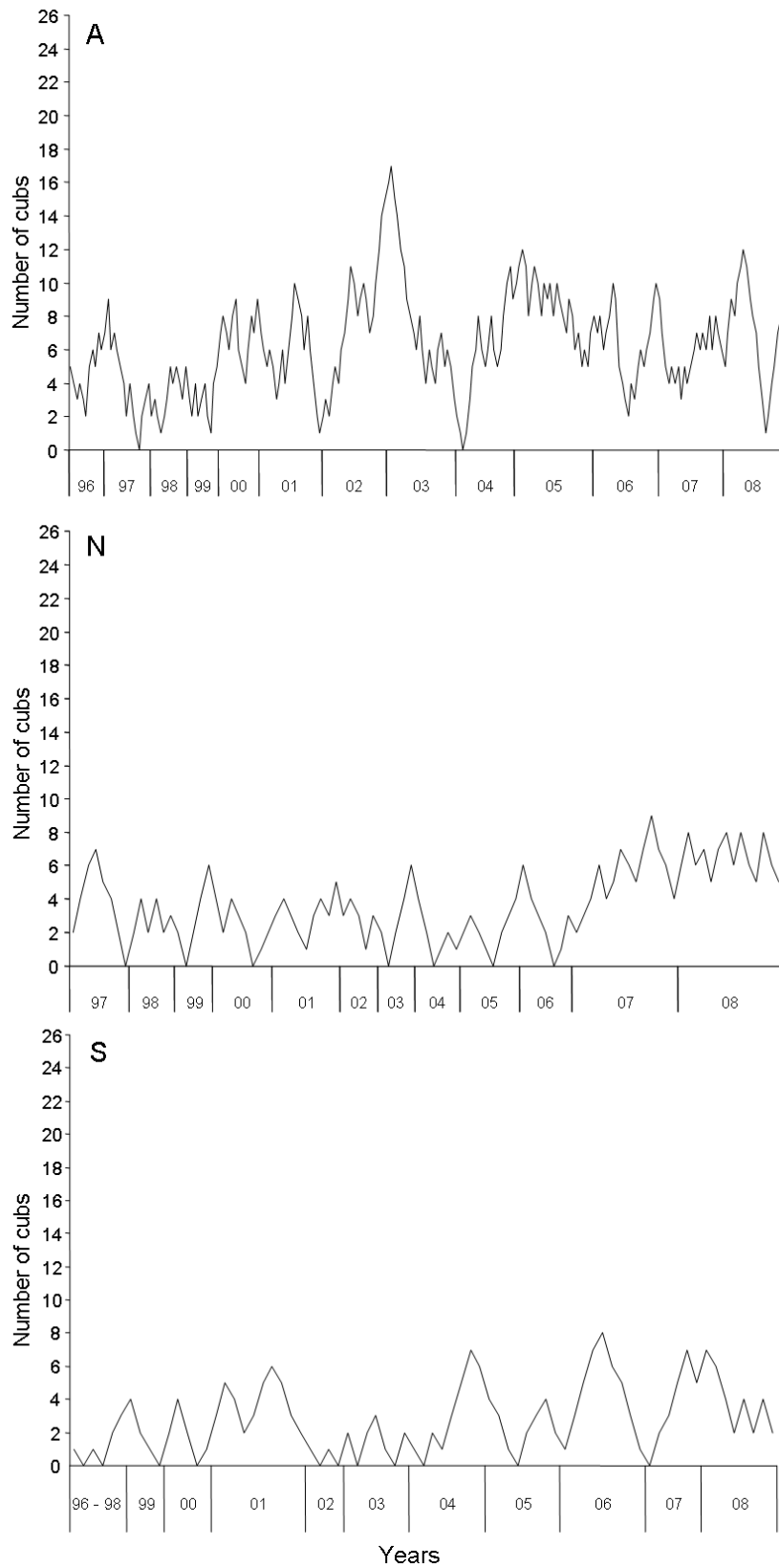


Figure 13: Absence and presence periods with increasing and decreasing numbers of cubs present in each clan across the whole observation time. Exemplary data of three clans in the NC with minimum number of absence periods (Clan A, 2 periods), medium number of absence periods (Clan N, 7 periods) and maximum number of absence periods (Clan S, 11 periods) are shown. Duration of periods is not displayed in this figure.

In the Crater subpopulation, young cubs were present during 61 periods (median duration of 310 days, range 8 – 3,218 days; see Fig. 14a) bridging intervals without cubs, whereas in the Serengeti clans, there were 8 such bridging intervals of a very long duration (median duration 3,149 days, range 275 – 3,997 days, see Fig. 14b). Young cubs were present for substantially longer periods in the Serengeti than in the Crater (Tarone-Ware $\chi^2 = 14.75$; d.f. = 1; $n = 69$; $p < 0.0005$).

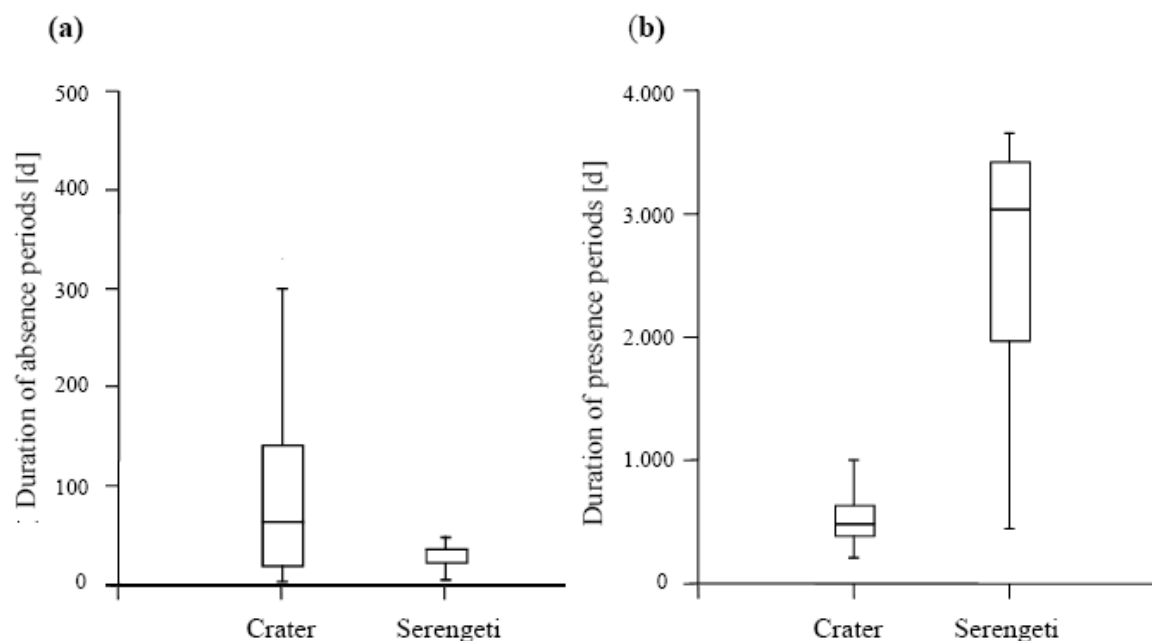


Figure 14: Differences in the duration of absence periods (a) and presence periods (b) in the Crater and the Serengeti hyena subpopulations. The box plots show distribution of the duration of periods in days for spotted hyena populations in the Crater and Serengeti ecosystem. The box indicates the interquartile range around the median (line inside box), and the vertical error bars represent values plus or minus 1.5 times the interquartile range.

3.4.4 DISCUSSION

In this Chapter I investigated infection patterns and the ability of persistence of CoV in a metapopulation of free-ranging spotted hyenas that is divided into two subpopulations, the Serengeti and the Crater subpopulation. RT-PCR results of a large sample size of faecal samples collected from individuals across different years living in both subpopulations revealed that there is no evidence of CoV shedding in the Crater subpopulation. In contrast, a moderate proportion (21.1 %, see Chapter 3.1) of positive faecal samples from individuals inhabiting the Serengeti were detected among hyena cubs, suggesting an age-specific infection pattern similar to FCoVs and CCoVs infecting domestic animals (de Groot & Horzinek 1995; Pratelli 2006). In domestic cats, the likelihood of CoV shedding may be associated with age as virus shedding decreases with age (Foley et al. 1997a,b). Results of experimental FCoV infections of cats showed that primary infections in young domestic cats led to a higher likelihood of

virus shedding than in secondarily infected older cats (Poland et al. 1996). This is consistent with the observed prevalence in this study where only few individuals (5.3 %, see Chapter 3.1) older than 12 months of age shed virus.

Pathogens have developed several mechanisms to persist in host populations. One is facilitating life-long infectivity of host individuals as in the case of FIV (Courchamp et al. 1995). A second possibility is to develop a state of latency in which the pathogen remains in the recovered host and may be shed at a later time again as known from CoV infection in domestic cats and domestic dogs where in some cases virus may be shed for short time periods, intermittently or continuously (Herrewegh et al. 1995; Addie & Jarrett 2001; Pratelli et al. 2001, 2002, 2004). I found no evidence of intermittent or continuous viral shedding. My results furthermore suggest that CoV infection in hyenas might induce life-long immunity and are therefore consistent with results previously reported by East et al. (2004). Even so, results presented in Chapter 3.1 demonstrate that Serengeti hyenas were infected by different FCoV and CCoV-like variants in different years, serological neutralisation assays tested by collaborators showed that antibodies against CoV from serum samples collected in different years neutralised TGEV, FCoV and CCoV (unpublished data), suggesting considerable cross-reactivity of antibodies developed in response to these different CoV variants. Such cross-reactivity could have induced immunity in hyenas against different CoV variants.

Spatial structuring of the host population may allow a pathogen to persist. If a population is divided into subpopulations, a pathogen may invade one subpopulation and then jump to another susceptible subpopulation leaving the original subpopulation to recover and to produce new susceptibles. As described by Hagenaars et al. (2004) the mean time for a virus to become extinct depends on social interactions between subpopulations. In this context the metapopulation of spotted hyenas composed of the Serengeti and Crater subpopulations could theoretically provide CoV with the opportunity to jump between the Serengeti and Crater subpopulations. This would require social contact between infected animals in one subpopulation contacting susceptible animals in another. Social activities between both hyena subpopulations occur relatively rarely and if so then mainly by dispersing adult males (Höner et al. 2005) whereas viral carriers are mainly restricted to den-bound cubs. Thus, the chance of transmission of CoV via rare dispersal events in either direction between the two subpopulations is likely to be low and thus is probably unlikely to permit viral persistence.

It is also possible to consider a sub-community structure within a metapopulation in terms of age classes or social classes, and in this case the social interactions between sub-communities of young versus old, or high versus low ranking animals may determine viral persistence.

When considering CoV persistence in the Serengeti subpopulation, the most important age class for viral persistence is cubs. Furthermore, the basic reproductive rate R_0 determined in this study was high among young cubs and decreased with increasing age in the clans of the Serengeti subpopulation, indicating that CoV has the potential to spread and be maintained in young spotted hyenas in the Serengeti subpopulation. When the rate of production of new susceptibles in a host population is low, pathogens may become extinct (Grenfell & Dobson 1995). In this study I found that a large section (38-46%) of susceptible hosts within the Serengeti subpopulation consisted of young cubs. When susceptible hosts are absent from the subpopulation, i.e. young cubs are absent for a period of time longer than the period of viraemia, then the most important demographic segment of the susceptibles is not present to support transmission, and thus pathogen extinction is possible. I determined the frequency and duration of absence periods of young cubs and detected that such periods occurred significantly more often and were substantially longer in the Crater subpopulation than in the Serengeti subpopulation. These results suggests that within the Crater subpopulation, the subcommunity structure based on age is highly likely to result in CoV extinction even after the virus successfully infected spotted hyenas on the Crater floor, as the main age class that supports transmission is frequently missing for periods longer than the estimated maximum infectious period of 68 days. The number of young cubs was generally higher in the Serengeti than in the Crater subpopulation and young cubs were present for substantially longer periods in the Serengeti subpopulation suggesting that their presence is likely to maintain viral persistence in the Serengeti. Even so, extinction of CoV may occur within any specific clan, should there be no new naïve young animals born within a group - an essential prerequisite to maintain transmission. The high seroprevalence of antibodies against CoV in the adult subcommunity would therefore act as a wall of protection against the reintroduction of infection to young cubs isolated at the den, and re-infection would only be possible as seroprevalence in adult clan members, in a clan with naïve cubs at the communal den, declines.

Not a single juvenile hyena from the Crater subpopulation was seropositive. However, three of five adult Crater spotted hyenas were seropositive in 2004 and two adults from 2004 and 2006 were seronegative. Although this is a small sample size, the results suggest high seroprevalence to CoV within the adult age class in the Crater subpopulation and CoV infection in the Crater subpopulation in previous years before 2004 after which it went extinct. As CoV possibly became extinct in the Crater subpopulation after 2004, it is likely that the cub and subadult age classes in the Crater were totally naïve, and thus infection could spread into this age classes in case CoV infection spread to the Crater or, should a susceptible Crater hyena be

infected with CoV when roaming outside the Crater it might have transmitted the virus to cubs at the communal den on its return. As animals born after viral extinction would be naïve, the virus would rapidly spread through susceptibles, as the ‘wall’ of exposed adults around communal dens would be weak, since a substantial proportion of adults would have entered the Crater subpopulation after the extinction of the virus.

There is evidence from serological investigations that lions inhabiting the Crater were exposed to CoV between at least 1984 and 1991 (Packer et al. 1999), suggesting that CoV variants might have been maintained in other carnivores in the Crater during that period of time. It remains unclear whether the CoV variant in other carnivores would be transmitted to spotted hyenas or whether other carnivores are involved in the persistence of the variants that infected spotted hyenas. It is currently unknown whether the CoV hyena variants occur in other carnivores (see Chapter 3.1). To answer this question, further characterisation of CoV variants from other species would be required.

In this Chapter I provide evidence of the presence of infectious spotted hyenas in the Serengeti and an absence of infectious spotted hyenas in the Crater during several years of non-invasive monitoring. I suggest this stark contrast in viral presence was caused by significant differences in the temporal presence of susceptibles between the Serengeti and Crater clans. The documented dispersal of males from the Serengeti to the Crater (Albert et al. 2000) was clearly insufficient to prevent the recent extinction of CoV in the Crater subpopulation as documented in this paper and a lack of recent virus transmission from infectious Serengeti individuals to Crater susceptibles. I suggest that immune adult spotted hyenas from the Serengeti subpopulation probably formed a ‘barrier’ to transmission between highly clustered subcommunities of infectious cubs stationed at communal dens in the Serengeti and that reinfection of the Crater subpopulation may occur in future. These results illustrate that pathogen dynamics within apparently similar and closely adjacent subpopulations may significantly differ as a result of variation in demographic stochasticity, and that viral traits adapted to large subpopulations may be less adapted to smaller subpopulations.

3.5 Characterisation of distinct *Hepatozoon* species infecting spotted hyenas and sympatric carnivores in the Serengeti ecosystem

3.5.1 INTRODUCTION

Apicomplexan hemogregarine *Hepatozoon* species are intracellular parasites with a life cycle that typically involves gametogenesis, fertilisation, and sporogony in a haematophagus invertebrate, and merogony followed by gamontogony in a vertebrate intermediate host. Infection of the vertebrate host normally occurs by ingestion of an infected haematophagus invertebrate host, mainly ticks (Smith 1996) but also fleas (Watkins et al. 2006). The primary tick vector for *H. canis* is suggested to be *Rhipicephalus sanguineus* (Craig 1990) but other ticks such as *Haemophysalis longicornus* or *Haemophysalis flavas* also act as vectors (Murata et al. 1995). The host tick for *H. americanum* appears to be *Amblyomma maculatum* (Mathew et al. 1998). In Africa the ticks *Rhipicephalus appendiculatus*, *Amblyomma habraeum* and *Amblyomma marmoreum* were suggested to act as vectors for *Hepatozoon* sp. in lions (Penzhorn et al. 1992) and *Rhipicephalus simus* for infection in hyenas (McCully et al. 1975).

Hepatozoon species are classified on the basis of morphologic characteristics, life history and host taxon, and recently molecular phylogenetic techniques were established to determine the evolutionary relationships among species (Vincent-Johnson et al. 1997; Mathew et al. 2000; Perkins & Keller 2001; Simpson et al. 2005, 2006; Criado-Fornelio et al. 2006). Globally, *Hepatozoon* infections in domestic and wild carnivore species have been attributed to *H. canis* or closely related undetermined species (Brocklesby & Vidler 1965; McCully et al. 1975; Conceição-Silva et al. 1988; Averbek et al. 1990; van Heerden et al. 1995), with the exception of the USA where *H. americanum* infects both domestic dogs (Vincent-Johnson et al. 1997) and wild carnivores (Kocan et al. 2000).

Hepatozoon gametocytes are commonly found incidentally in blood smears of domestic dogs and free-ranging carnivores such as lions, leopards *Panthera pardus*, cheetahs, silver-backed jackals, spotted hyenas and even large-spotted genets *Genetta tigrina* (Brocklesby & Vidler 1963, 1965; Keep 1970; McCully et al. 1975; Averbek et al. 1990; Penzhorn et al. 1992). A recent study that applied genetic techniques found that 42.3% of free-roaming domestic dogs in Sudan, north-east Africa, tested positive for *Hepatozoon* sp. infection (Oyamada et al. 2005). Phylogenetic analysis showed that the *Hepatozoon* species detected in these dogs were closely related or identical to *H. canis* (99-100% sequence similarity). *Hepatozoon* gametocytes found in blood smears of African carnivores are often called *H. canis* irrespective of the

host species, and various named *Hepatozoon* species from other host species have been lumped under '*H. canis*' (Levine 1988). Clear morphological differences of *Hepatozoon* gametocytes found in cheetahs and African wild dogs, respectively, suggest that the assumption that *Hepatozoon* in any African carnivore host must be *H. canis* may be false (Peirce et al. 1995).

Little is known about the phylogenetic relationship between different isolates of *Hepatozoon* sp. that infect African wild carnivores. Even when molecular techniques were applied to determine infection, isolates were not sequenced so that the *Hepatozoon* species involved remains unknown. For example, two of 301 blood samples from African wild dogs in South Africa were positive for *Hepatozoon* sp. using PCR and subsequent reverse line blot hybridisation but no sequence analysis was conducted to determine the phylogenetic relationship of the *Hepatozoon* species involved (Matjila et al. 2008).

Colleagues and I have previously identified a distinct *Hepatozoon* sp. by histopathological and molecular analysis in spotted hyenas in the Serengeti National Park that was closely related to, but still distinct from, a *Hepatozoon* variant previously detected in domestic cats in Spain (Criado-Fornelio et al. 2006). *Hepatozoon* infection was directly responsible for the death of two of the 11 infected juvenile spotted hyenas in that study, suggesting that heavy *Hepatozoon* infection is an important source of mortality in young animals (East et al. 2008). I extended our work and investigated further blood and tissue samples from spotted hyenas from the Crater and Serengeti subpopulation for the presence of *Hepatozoon* and examined the phylogenetic relationship of the sequences obtained by applying PCR methods that allowed sequence analysis of a larger fragment of the genome. Additionally, I examined samples collected from sympatric carnivores living in close proximity to hyenas from both populations.

In the Serengeti ecosystem there are 26 carnivore species belonging to six families of carnivores (Sinclair & Arcese 1995). This study included 11 carnivore species from five different families, the Hyaenidae, Canidae, Felidae, Viverridae and Herpestidae inhabiting the Serengeti National Park, the adjacent Ngorongoro Crater and the Loliondo Game Controlled Area close to the north-eastern border of the Serengeti National Park (see Fig. 1, Chapter 2). Severe infestations of ticks on wild animals were previously reported from the Ngorongoro Crater and altogether 15 tick species were identified from the Crater floor, including the potential *Hepatozoon* tick vector *Rhipicephalus sanguineus*, *Rhipicephalus appendiculatus* as well as other *Rhipicephalus* and *Amblyomma* species (Fyumagwa et al. 2009).

To obtain a better understanding of the evolutionary phylogenetic relationship of the obtained *Hepatozoon* variants within the carnivore guild in the Serengeti ecosystem in order to improve our understanding of pathogen within-guild transmission, I used two published primer pairs to obtain a longer fragment of the previously published *Hepatozoon* species infecting hyenas and designed *Hepatozoon*-specific primer pairs for use during routine screening of samples from numerous carnivore species.

3.5.2 METHODS

Sample collection

I screened a total of 85 blood ($n = 38$) and tissue ($n = 47$) samples from individual spotted hyenas including 36 cubs and 48 individuals older than 12 months of age (of one animal the age was unknown; this animal was excluded from statistical analysis) inhabiting the Serengeti National Park, and 19 samples from individual spotted hyenas, including 5 cubs and 14 individuals older than 12 months of age, from the Ngorongoro Crater. Additionally I screened 46 blood and tissue samples from 11 sympatric carnivore species (38 samples obtained from the Serengeti, 6 samples from the Loliondo Game Controlled Area, 2 samples collected in the Ngorongoro Crater) belonging to the families Canidae, Felidae, Viverridae and Herpestidae (see Table 1, Chapter 2). Samples were obtained either from animals that died from natural causes or from animals that were immobilised. Blood and tissue samples were stored as described in Chapter 2. The samples obtained in the Loliondo Game Controlled Area were collected from African wild dogs that died of canine distemper virus infection close to the north-eastern border of the Serengeti National Park (Goller et al. 2010; see also Chapter 3.6).

PCR screening and genetic phylogeny

All primer pairs used in this study target regions of the 18S rRNA of *Hepatozoon*. For screening I used our recently published primer pairs HEMO 3 and HEMO 4 that amplify a 462 nucleotide (nt) long fragment (East et al 2008) and additionally designed a specific *Hepatozoon* primer pair HEMO 9 (5'-TTA TAA CCT TGG CTG GTA AGC-3') and HEMO 10 (5'-TAT CAC TTA GAC GAA GGA GAA GT-3') that amplify a 266 nt fragment and revealed best results with an annealing temperature of 58°C. Samples that revealed PCR products with both primer pairs were considered as positive. To amplify a longer fragment useful for phylogenetic analysis I used the published primer pair BT1 (forward) and BT2 (reverse) that amplify a 430 nt fragment (Criado-Fornelio et al. 2003), and the primer pair Hep-1 (f) and Hep-2 (r) that amplifies a 666 nt fragment (Criado-Fornelio et al. 2006). Both primer pairs cover a total

length of approximately 750 nt. Fragments were sequenced as described in Chapter 2 and compared with published *Hepatozoon* sequences (Fig. 15) The complete alignment included 22 published sequences in addition to the sequences obtained from spotted hyenas and sympatric carnivores in this study. Accession numbers of published sequences used in the phylogenetic study are included in the phylogenetic tree (Fig. 15) which was generated using a neighbourjoining method with maximum composite likelihood distances as implemented in Mega 4 (Tamura et al. 2007). All sequences generated and used for phylogenetic analyses are contained in Appendix D.

Statistics

I performed log-likelihood ratio tests to examine differences in the prevalence of infection in both subpopulations and differences in prevalence in both age groups.

3.5.3 RESULTS

Prevalence of infection

Both screening primer pairs (HEMO3/4 and HEMO9/10) revealed PCR products of the expected lengths from 75 Serengeti individuals (89.3 % positive of 84 individuals; Table 16). From the 48 individuals older than 12 months of age, 43 were infected (89.6 % positive) with *Hepatozoon*. In the samples of 36 cubs pathogenic DNA was present in 32 (88.9% positive). In the Crater hyenas, 84.2 % of 19 individuals (Table 16) were positive, including 4 of 5 cubs (80.0 % positive) and 12 of 14 older individuals (85.7 % positive). There was no difference in infection prevalence between the Serengeti and Crater spotted hyena subpopulation (log-likelihood $\chi^2 = 0.16$; d.f. = 1; n = 103; p = 0.53) and cubs of both subpopulations were as likely to be infected as older individuals (log-likelihood $\chi^2 = 0.02$; d.f. = 1; n = 103; p = 0.90).

Prevalence of *Hepatozoon* infection of sympatric carnivores was high in African wild dogs from the Loliondo Game Controlled Area (100% infected, 6 individuals from one pack, Goller et al. 2010), in lions inhabiting the Serengeti National Park (90.9 % infected) and low in Serengeti bat-eared foxes (15.4% infected). Sample sizes of other sympatric carnivore species were too low to determine reliable prevalences (Table 16). I could not detect *Hepatozoon* DNA in samples of the side-striped jackal, silver-backed jackal, serval cat, slender or dwarf mongoose. In the Crater, the one lion sample that was screened was positive, and one sample from a silver-backed jackal was negative (Table 16).

Table 16: Prevalence of Hepatozoon infection in spotted hyenas and sympatric carnivores in the Serengeti National Park (SNP), the Ngorongoro Crater (NC) and the Loliondo Game Controlled Area.

Family	Species	Proportion positive [%] (number positive/total)	
		SNP	NC
Hyaenidae	Spotted hyena	89.3 (75/84)	84.2 (16/19)
Canidae	bat-eared fox	15.4 (2/13)	-
	silver-backed jackal	0 (0/2)	0 (0/1)
	side-striped jackal	0 (0/1)	-
	African wild dog*	100 (6/6)	-
Felidae	serval cat	0 (0/2)	-
	lion	90.9 (10/11)	100 (1/1)
Viverridae	common genet	50.0 (2/4)	-
Herpestidae	white-tailed mongoose	66.0 (2/3)	-
	slender mongoose	0 (0/2)	-
	Dwarf mongoose	0 (0/1)	-

* samples obtained in the Loliondo Game Controlled Area 12km from the Serengeti National Park

Phylogenetic analysis

I sequenced PCR products obtained with primer pairs BT1/BT2 and Hep1/Hep2 from 10 infected spotted hyenas from the Serengeti National Park of which seven sequences were identical and grouped as ‘HY SNP’ in Fig. 15. All other sequences obtained from spotted hyenas were labelled as follows: code HY followed by animal ID (e.g. P126) and the abbreviation SNP if from the Serengeti National Park and NC if from the Ngorongoro Crater. From Serengeti sympatric carnivores I sequenced PCR products from one lion (labelled LI 01 SNP), one common genet (GE 16 SNP), one bat-eared fox (FX 23 SNP) and one white-tailed mongoose (WTM 14 SNP). From the Loliondo Game Controlled Area close to the Serengeti National Park I sequenced positive PCR products obtained from samples of two African wild dogs (AWD Lpi6499 LGCA and AWD Lpi6514 LGCA). From the Ngorongoro Crater I sequenced samples from five spotted hyenas of which two were identical and labelled as ‘HY NC’ in Fig. 15 and one sequence was obtained from a lion (LI 02 NC).

All sequences obtained from spotted hyenas and sympatric carnivores in the Serengeti National Park and the Ngorongoro Crater were most similar to a published variant termed

‘*Hepatozoon* sp. “felis” Spain 2’ that was recently obtained from domestic cats in Spain (Criado-Fornelio et al. 2006). Similarity ranged between 95.1 and 99.3 % nt similarity whereas the sequences obtained from the African wild dogs in the Loliondo Game Controlled Area where most similar to variant ‘*Hepatozoon* “canis” Spain 2’, previously detected in infected domestic dogs in Spain (Criado-Fornelio et al. 2006) with 95.1 and 98.6 % nt similarity respectively (Table 17).

Table 17: Similarity of the *Hepatozoon* variants obtained from spotted hyenas and sympatric carnivores in the Serengeti National Park (SNP), the Loliondo Game Controlled Area (LGCA) and the Ngorongoro Crater (NCA) to published sequences from elsewhere. In bold: similarities referred to in the main text.

<i>Hepatozoon</i> sp.	SNP								LGCA		NCA				
	HY SNP	HY P162	HY P279	HY Z94	HY FX23	WTM 14	GE 16	LI 01	AWD Lpi6499	AWD Lpi6514	HY A84	HY A163	HY NC	HY F42	LI 02
HY SNP	100	99.0	99.5	99.4	99.1	95.2	98.1	99.3	90.7	94.2	99.0	100	98.9	98.9	96.7
HY P162 SNP		100	98.6	99.3	99.0	95.1	97.9	99.1	90.5	94.0	99.7	99.0	99.8	97.9	96.6
HY P279 SNP			100	99.0	98.7	94.8	97.7	98.9	90.3	93.8	98.6	99.5	98.5	98.7	96.3
HY Z94 SNP				100	99.4	95.5	98.3	99.5	90.9	94.4	99.3	99.4	99.1	98.6	97.0
FX 23 SNP					100	95.2	98.2	99.5	90.9	94.4	99.0	99.1	98.9	98.1	97.0
WTM 14 SNP						100	95.8	95.4	90.1	92.7	95.1	95.2	95.0	94.2	96.3
GE 16 SNP							100	98.2	91.6	95.1	97.9	98.1	97.8	97.0	97.4
LI 01 SNP								100	90.8	94.3	99.1	99.3	99.0	98.2	97.1
AWD Lpi6499 LGCA									100	95.5	90.5	90.7	90.4	89.7	90.9
AWD Lpi6514 LGCA										100	94.0	94.2	93.9	93.2	94.6
HY A84 NC											100	99.0	99.8	97.9	96.6
HY A163 NC												100	98.9	98.9	96.7
HY NC													100	97.8	96.5
HY F42 NC														100	95.6
LI 02 NC															100
<i>H. felis</i> Spain1	98.7	98.9	98.3	99.0	99.0	95.0	97.7	99.1	90.3	93.8	98.7	98.7	98.7	97.7	96.6
<i>H. felis</i> Spain2	98.9	99.0	98.5	99.1	99.1	95.1	97.8	99.3	90.4	93.9	98.9	98.9	98.9	97.8	96.7
<i>H. canis</i> Spain1	94.3	94.2	93.9	94.6	94.6	92.7	94.8	94.4	95.0	98.5	94.2	94.3	94.0	93.4	94.4
<i>H. canis</i> Spain2	94.4	94.3	94.0	94.7	94.7	92.5	94.8	94.6	95.1	98.6	94.3	94.4	94.2	93.5	94.3
<i>H. americanum</i> ¹	88.4	88.4	88.0	88.6	88.3	88.6	88.8	88.4	90.6	86.8	88.4	88.4	88.3	87.4	89.6
<i>H. sp.</i> Curupira2	93.0	93.0	92.6	93.1	92.9	92.1	93.4	93.0	87.5	90.9	93.0	93.0	92.9	91.9	94.0
<i>H. canis</i> Curupira3	94.4	94.3	94.0	94.7	94.7	92.8	95.0	94.6	95.0	98.6	94.3	94.4	94.2	93.5	94.6
<i>H. canis</i> Venezuela1	94.4	94.3	94.0	94.7	94.7	92.5	94.8	94.6	95.1	98.6	94.3	94.4	94.2	93.5	94.3
<i>H. canis</i> Venezuela2	94.6	94.4	94.2	94.8	94.8	92.7	95.0	94.7	95.1	98.6	94.4	94.6	94.3	93.6	94.4
<i>H. ayorgbor</i>	93.0	92.9	92.6	93.1	92.9	92.1	93.4	93.0	87.5	91.0	92.7	93.0	92.7	92.1	93.4
<i>H. sp.</i> Boiga	89.2	89.1	89.2	89.4	89.1	88.2	89.4	89.2	84.5	87.5	89.2	89.2	89.2	88.6	89.4
<i>H. sp.</i> HepBiCM001	92.7	92.6	92.3	92.9	92.6	91.5	93.1	92.7	87.5	91.0	92.5	92.7	92.5	91.8	92.9
<i>H. sp.</i> BV1	91.7	91.8	91.3	91.8	91.5	90.7	92.3	91.7	86.6	90.1	91.7	91.7	91.7	90.7	91.9
<i>H. sp.</i> BV2	91.9	92.1	91.5	92.1	91.8	91.0	92.3	91.9	86.6	90.1	91.9	91.9	91.9	91.0	92.2
<i>H. canis</i> 08 Sudan	81.8	81.6	81.4	82.0	82.0	81.5	82.5	82.0	89.3	85.4	81.6	81.8	81.5	80.8	81.7
<i>H. canis</i> 47/60 Sudan²	80.2	80.0	79.8	80.4	80.4	80.1	81.0	80.4	87.9	84.1	80.0	80.2	79.9	79.2	80.2
<i>H. canis</i> 78 Sudan	80.1	79.9	79.7	80.3	80.3	79.8	80.6	80.3	87.5	83.7	79.9	80.1	79.8	79.1	79.8
<i>H. sipedon</i>	20.5	20.8	20.5	20.5	20.6	20.9	20.5	20.6	20.5	19.7	20.6	20.5	20.8	20.4	20.5
<i>H. catesbiana</i>	20.1	20.4	20.1	20.1	20.2	20.5	20.1	20.2	20.4	19.5	20.2	20.1	20.4	20.0	20.2
<i>H. americanum</i> 3	20.2	20.6	20.2	20.4	20.5	20.8	20.4	20.5	20.4	19.5	20.5	20.2	20.6	20.1	20.4

¹ *H. americanum* AF176836

² *H. canis* 47 Sudan and *H. canis* 60 Sudan were identical

³ *H. americanum* AF206671

The similarity of all variants obtained from wild carnivores in Tanzania in this study with sequences obtained from domestic dogs in Sudan in north-east Africa was low and ranged between 79.1 and 89.3 % (Table 17). Comparison of *Hepatozoon* fragments from spotted hyenas in the Serengeti National Park revealed that the seven identical sequences (HY SNP) were also identical to a variant obtained from a Crater hyena (HY 163 NC) and other variants from Serengeti spotted hyenas had a close similarity to variants infecting spotted hyenas in the Crater (between 98.1 and 99.7 % nt similarity). The sequences obtained from other sympatric carnivores in the Serengeti National Park, including a bat-eared fox, a white-tailed mongoose, a common genet and a lion, differed from those obtained from spotted hyenas and nt similarities ranged between 94.8 and 99.5 % (Table 17).

The phylogenetic analysis revealed that the *Hepatozoon* fragments from spotted hyenas in the Serengeti National Park and Crater clustered together with variants obtained from a bat-eared fox, a lion and a common genet in the Serengeti National Park (Fig. 15). Included in this cluster were two *Hepatozoon* variants previously described from domestic cats in Spain (Criado-Fornelio et al. 2006). Within this large cluster, the two identical sequences (HY NC) and the sequence HY A84 NC from Crater hyenas as well as one sequence from a hyena in the Serengeti (HY P162 SNP) formed a highly supported (bootstrap value 96) sub-cluster indicated as ‘hyena 1’ in Fig. 15. Other variants obtained from hyenas in the Serengeti and Crater formed another sub-cluster (‘hyena 2’, with a lower bootstrap value of 77) and the variant HY Z94 SNP from the SNP was separated from sub-cluster ‘hyena 2’ (Fig. 15). Separated from this large cluster of variants from a mixture of host species in both the Serengeti National Park and Crater were two further wildlife variants, one from a white-tailed mongoose in the Serengeti and another from a lion in the Crater (Fig. 15). The sequence from the Crater lion as well as from the white-tailed mongoose clustered with *H. americanum*, a *Hepatozoon* variant known to infect domestic and wild canids in the USA (Mathew et al. 2000) and *H. sp. Curupira 2* described from wild crab-eating foxes (*Dusicyon thous*) in Brazil (Criado-Fornelio et al. 2006).

Unlike the *Hepatozoon* variant from the bat-eared fox in SNP, the other canid host species included in my analysis, i.e. the African wild dog, was infected with a *Hepatozoon* sp. not found in any other wild carnivore species within either the Serengeti National Park or the Crater. The African wild dogs were infected with a variant (Fig. 15; cluster ‘AWD’) most similar to variants of *H. canis* but distinct from the only other *H. canis* variant described from Africa in the Sudan.

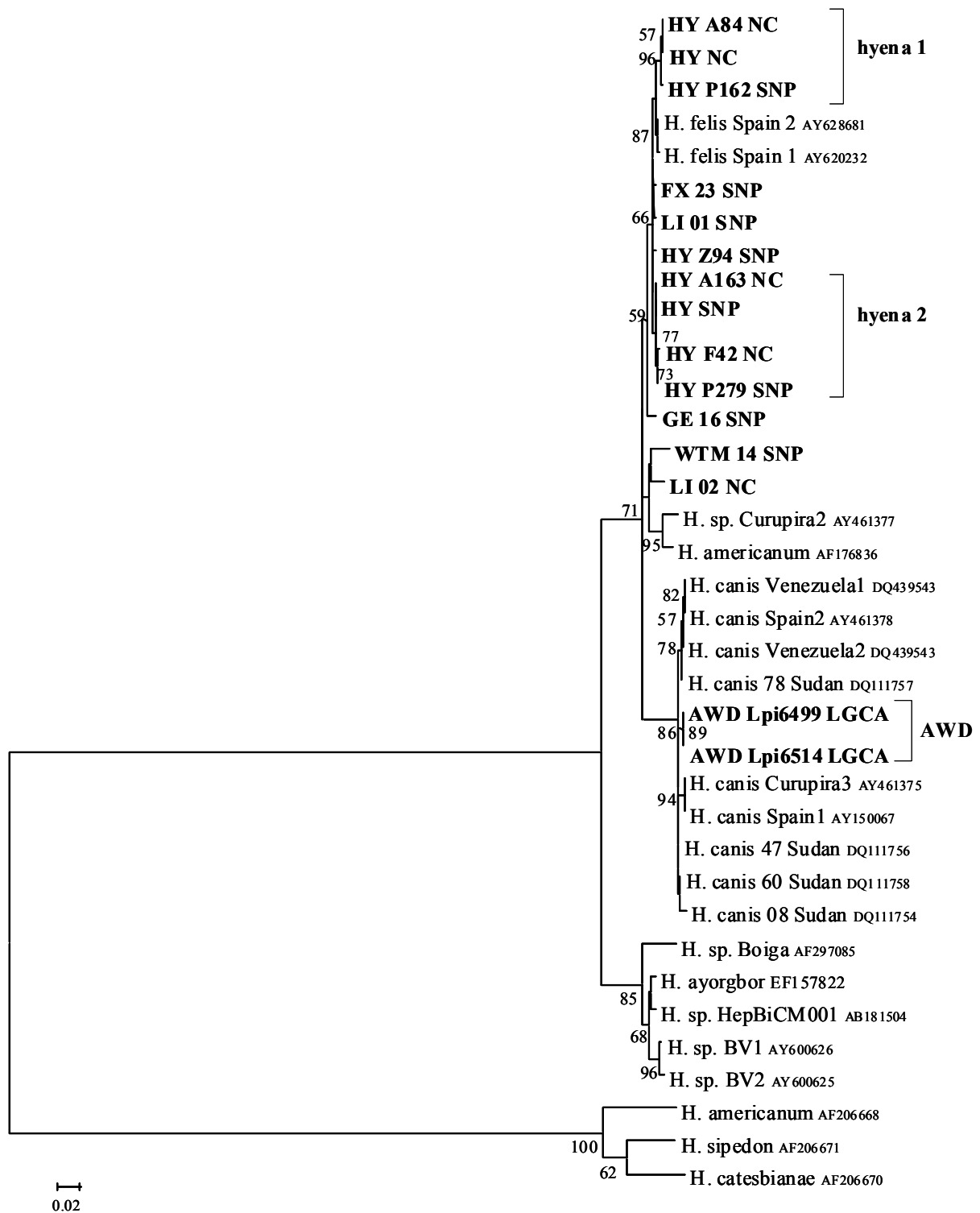


Figure 15: Phylogenetic relationship of *Hepatozoon* sequences obtained from spotted hyenas and sympatric carnivores in the Serengeti National Park (SNP), the Ngorongoro Crater (NC) and the Loliondo Game Controlled Area (LGCA) in Tanzania, in relation to published *Hepatozoon* sequences based on a segment of the 18S rRNA gene. Accession numbers of published sequences are indicated.

3.5.4 DISCUSSION

These results suggest that the carnivore guild in the Serengeti ecosystem was infected with a remarkable diversity of *Hepatozoon* variants, including at least two distinct *Hepatozoon* ‘types’ (‘hyena 1’ and ‘hyena 2’ in Fig. 15) infecting spotted hyenas, and possibly another three *Hepatozoon* ‘types’ infecting sympatric carnivores.

Spotted hyenas in the Serengeti National Park and Ngorongoro Crater as well as a bat-eared fox, a genet and a lion in the Serengeti National Park were infected with *Hepatozoon* variants most similar (between 95.1 and 99.3% nt similarity, Table 17) to ‘*Hepatozoon* “felis” Spain 2’, a variant previously detected in domestic cats in Spain (Criado-Fornelio et al. 2006), and a result similar to that obtained by East et al. (2008). These sequences clustered together and may thus represent a clade of *Hepatozoon* variants infecting a diversity of wild carnivores including members of the Hyaenidae, Felidae, Canidae and Viverridae, suggesting that the variant detected in cats in Spain is not specialized to infect felids but rather a broad spectrum of wild carnivore species, and may also be prevalent in Spanish wildlife carnivore species, including the common genet which has extended its range into Spain, or the Iberian lynx (*Lynx pardinus*). Sequences obtained from seven Serengeti hyenas (HY SNP) and one Crater hyena (HY A163 NC) were identical and are thus likely to represent one distinct *Hepatozoon* species (‘hyena 2’ in Fig. 15). In the phylogenetic analysis these sequences formed a sub-cluster with two other sequences (HY F42 NC and HY P279 SNP) suggesting, that these two sequences are similar variants of the same *Hepatozoon* species/type that circulates in the Serengeti ecosystem. Two sequences obtained from Crater hyenas were identical (labelled as ‘HY NC’ in Fig. 15 and Table 17) and highly similar to another variant obtained from a Crater hyena and one Serengeti hyena. They formed a well supported sub-cluster and may thus belong to another distinct *Hepatozoon* species/type (‘hyena 2’, Fig. 15). One hyena sequence (HY Z94 SNP) did not cluster within those two *Hepatozoon* ‘hyena types’ but was also not significantly supported as a separate branch and therefore might represent another variant of *Hepatozoon* species (Fig. 15).

The sequences of *Hepatozoon* obtained from a white-tailed mongoose from the Serengeti National Park as well as a lion from the Ngorongoro Crater were most closely related to *H. americanum* and *H. sp. Curupira 2*. The high bootstrap value of 95 suggests that these variants in African carnivores were distinct from ‘*H. americanum* AF176836’ and ‘*H. sp. Curupira 2*’ and a low (< 50; not shown in the Fig. 15) bootstrap value suggests that the sequence obtained from the white-tailed mongoose and the lion may differ only a bit.

In contrast, two sequences obtained from African wild dogs in the Loliondo Game Controlled Area were most similar to *H. canis* 'Spain 2' and formed a sub-cluster within the *H. canis* group. Both sequences were sufficiently different from all other sequences from wild Serengeti / Ngorongoro carnivores examined in this chapter, to suggest that the *Hepatozoon* variant infecting these African wild dogs was distinct.

The low similarity of the sequences obtained from carnivores in this study to *Hepatozoon* detected in domestic dogs from Sudan (Oyamada et al. 2005) suggests that *Hepatozoon* species may vary across different geographic regions in Africa. As already pointed out by Pierce et al. (1995), who found morphological differences in *Hepatozoon* gametocytes obtained from cheetahs and African wild dogs, *Hepatozoon* gametocytes detected on blood smears in African carnivores are often wrongly classified as *H. canis*. The genetic diversity of *Hepatozoon* obtained in this study supports this suggestion. The results demonstrate that *Hepatozoon* variants infecting Serengeti carnivores were distinct from published variants and suggest that a high diversity of *Hepatozoon* strains circulate in Tanzanian carnivores. My results demonstrate the value of molecular techniques to investigate the evolutionary relationship between *Hepatozoon* species. It is likely that more extensive screening will reveal an even greater diversity of *Hepatozoon* variants in the carnivore community in the Serengeti ecosystem.

All *Hepatozoon* variants obtained from Serengeti and Crater carnivores were distinct from the *H. canis*-like variant that infected African wild dogs in the Loliondo Game Controlled Area. My data are insufficient to clarify this difference in these areas. A possible explanation for this distinction might be that the Loliondo Game Controlled Area is outside the Serengeti National Park (see Fig. 1; Chapter 2) and inhabited and used by Maasai pastoralists plus their livestock and domestic dogs that come from further east. African wild dogs that inhabit this area might share the same host tick species with domestic dogs and thus might become infected with similar *Hepatozoon* species that may also infect domestic dogs. In contrast, in the Serengeti National Park domestic dogs are not allowed. Thus, sympatric carnivores within the Serengeti National Park may maintain other *Hepatozoon* variants transmitted by specific tick vectors, than carnivores outside the park. However, this suggestion is contradicted by the results from the Ngorongoro Crater which is also frequently used by Maasai pastoralists, their livestock and domestic dogs because the *Hepatozoon* variants detected in Crater carnivores were similar to the variants detected in the Serengeti.

Within the spotted hyena population, the prevalence of *Hepatozoon* infection was similarly high in the Serengeti (89.3 % positive) and the Crater subpopulation (84.2 % positive). High prevalence of infection was also observed in Serengeti lions, consistent with observations

made by Averbeck et al. (1990) who reported *Hepatozoon* infection in blood smears obtained from all 123 lions living in the Serengeti National Park. Prevalence of infection was also high in African wild dogs, with all of 6 tested wild dogs being infected in the Loliondo Game Controlled Area. As these six animals were members of a single pack of wild dogs it is likely that tick hosts were shared via allogrooming, explaining the high *Hepatozoon* prevalence. Carnivores groom ticks off their own coats and social carnivores such as spotted hyenas, lions and African wild dogs often allogroom group members. They could thus become infected by ingesting infected ticks or hepatozoal oocysts that might be released when the ticks are damaged by the animal's teeth. But grooming behaviour is not necessarily the only route of *Hepatozoon* transmission. Most carnivores are predatory and feed on a wide variety of vertebrates, including birds and mammals. The suggested ticks that harbour *Hepatozoon* (e.g. *R. appendiculatus*) are so called 'three-host-ticks' which need three blood meals during their life. The first blood meal is taken before the larva moults into the nymph, another meal is needed before the nymph moults into the adult stage where they take a final blood meal after which females will lay their eggs (Walker et al. 2003). For each blood meal, ticks may feed on different host species and hosts. The tick species *R. appendiculatus* for example can be found on scrub hares *Lepus saxatilis* or smaller antelopes as well as on carnivore species (Walker et al. 2003). Thus, prey animals may carry ticks, some of which may be infected with *Hepatozoon*. It is therefore likely that wild herbivores which serve as prey for Serengeti and Crater carnivores share the same ticks as their predators. Tick hosts of *Hepatozoon* variants that are attached to prey might be ingested by carnivore predators whilst feeding and thus *Hepatozoon* transmission would be possible. Such a prey-associated *Hepatozoon* transmission could explain why the African wild dogs were infected by a completely different variant in comparison to Serengeti and Crater carnivores. African wild dogs inhabiting the Loliondo Game Controlled Area do consume livestock, particularly goats, which is probably infested with ticks that carry other *Hepatozoon* species than wild herbivores. Although the Maasai pastoralists frequently enter the Crater with their livestock it is very unlikely that Crater carnivores feed on it regularly as the livestock is usually well protected by the Maasai.

Prey-associated *Hepatozoon* transmission could also explain the low prevalence of infection in bat-eared foxes. Bat-eared foxes are nearly exclusively insectivorous and predominantly feed on termites (Mackie & Nel 1989; Maas 1993, 1994). A minor part of their food intake consists of small rodents, birds and eggs. Thus the chance to acquire infection by consuming tick-infested prey is low and could explain the low prevalence of infection.

The definite tick vectors of the *Hepatozoon* species described in this study are unknown. The tick *Rhipicephalus sanguineus* is an important vector of *H. canis* worldwide (Baneth et al. 2001) and oocysts of *Hepatozoon* have been reported in other tick species found on carnivores in Africa, including *Rhipicephalus simus* (McCully et al. 1975). A previous study (Fyumagwa et al. 2009) on tick abundance in the Ngorongoro Crater revealed several tick species, including both *Rhipicephalus* species as well as other species belonging to the genera *Rhipicephalus* and *Amblyoma*. It is likely that these other tick species might also act as vectors for the different *Hepatozoon* species described in this Chapter. To obtain a better understanding of potential tick hosts for different *Hepatozoon* species infecting carnivores in the Serengeti National Park, the Ngorongoro Crater and the Loliondo Game Controlled Area, tick surveys should be conducted in the respective locations and the collected ticks should be investigated by molecular biological methods for the presence of *Hepatozoon* variants.

In summary, my results suggest the presence of a high diversity of different *Hepatozoon* species infecting the Serengeti carnivore guild. The results furthermore show that spotted hyenas are likely to become infected with more than one *Hepatozoon* species. *Hepatozoon* species infecting carnivores in the Serengeti and Ngorongoro Crater formed a distinct cluster and were different from a *Hepatozoon* species infecting African wild dogs in the adjacent Loliondo Game Controlled Area. I suggest this difference resulted from ingesting ticks from killed livestock in the Loliondo Game Controlled Area. Prevalence of infection was high in hyenas, lions and wild dogs but rather low in bat-eared foxes. I suggest this discrepancy was a result of the insectivorous diet of bat-eared foxes which are thus less likely to experience prey-associated tick ingestion.

Further screening of ticks from carnivores and potential prey species for the presence of *Hepatozoon* is required to obtain a better understanding of transmission of *Hepatozoon* within the carnivore guild and between prey and carnivores. Additional samples from other carnivores should be screened to establish prevalence of infection in sympatric carnivores. This is the first description of the occurrence of distinct *Hepatozoon* species based on phylogenetic sequence analysis which infect free-ranging members of African carnivores belonging to the

3.6 Fatal canine distemper infection in a pack of African wild dogs (*Lycaon pictus*) in the Serengeti ecosystem, Tanzania

3.6.1 INTRODUCTION

Although diseases are listed as one of the top five causes of extinction, currently there is insufficient information to determine the level of threat diseases pose to the viability of many wildlife populations (Smith et al. 2006). The African wild dog is an endangered species that has declined in numbers throughout much of its former range, mostly due to habitat loss and human persecution (Fanshawe et al. 1991). The African wild dog is a cooperative breeder that lives in tightly bonded social packs (Moehlmann & Hofer 1997). African wild dogs have been studied in several African countries but despite this large research effort the impact of diseases on the population dynamics of this species is unclear (Fanshawe et al. 1991; Burrows et al. 1994; Creel et al. 1997; Alexander et al. 2010).

In this Chapter I report an outbreak of disease in a pack of free-ranging African wild dogs in the Loliondo Game Controlled Area, 12 km from the northeastern boundary of the Serengeti National Park, Tanzania (see Fig. 16). I describe clinical signs, internal pathologic changes, and the identification of CDV as the primary causative agent of mortality in the pack members examined. I provide genetic sequence data for a fragment of the relatively conserved phosphoprotein (P) gene and the more variable fusion (F) gene from the CDV variant that infected the pack.

CDV infection causes reduced immune function in hosts (von Messling et al. 2004) which is likely to increase the susceptibility of CDV infected hosts to further infection by other pathogens (Beldomenico et al. 2008). Pre-existing infections are also thought to influence the clinical course of morbillivirus infections (Akineden et al. 2005; Munson et al. 2008). Colleagues and I therefore screened the samples for the presence of the following pathogens: *Babesia* sp. because severe infection with this tick-borne blood parasite is suggested to increase expression of clinical disease in CDV infected lions (Munson et al. 2008); *Hepatozoon* sp. because infection with this tick-borne blood parasite may impair immune function, especially in young hosts (East et al. 2008; see also Chapter 3.5), and because prevalence of *Hepatozoon* sp. infection can be high in African wild dog populations (van Heerden et al. 1995, see also Chapter 3.5); *Streptococcus equi* subsp. *ruminatorum* because this bacterium was identified as a concurrent infection during a mass epidemic of phocine distemper virus (PDV) in European harbour seals (Akineden et al. 2005) and was the likely causative agent of a recent disease outbreak in spotted hyenas (Höner et al. 2006; Speck et al. 2008) in the Ngorongoro

Conservation Area (Fig. 16); rabies virus because all tested African wild dog carcasses from the Serengeti ecosystem between 1989 and 1992 were rabies positive (Burrows et al. 1994; East & Burrows 2001; Woodroffe 2001); and canine parvovirus (CPV) which is an important and comparatively recent disease of canids (Parrish & Kawaoka 2005) that is known to have infected African wild dogs in the Serengeti ecosystem (Burrows et al. 1994).

3.6.2 METHODS

Histopathology

Tissue samples from six relatively fresh carcasses (four pups, two adults) including liver, spleen, lung, heart, brain and kidney were stored in 10% neutral buffered formalin embedded in paraffin, sectioned at 4µm and stained with hematoxylin eosin. For immunohistochemistry tissue sections were deparaffinized and after antigen retrieval (in a pressure cooker for 2 min in 0.01 m citrate buffer, pH 6.0) slides were processed in an automated immunohistochemistry slide stainer (DAKO, Hamburg, Germany) through the following steps: 30 min incubation with a 1:3000 dilution of mouse anti-canine-distemper-virus antibodies (AbD Serotec MCA2538H, Serotec, Düsseldorf, Germany); washing with Tris-HCl buffer; 20 min incubation with biotinylated secondary antibody (Zyto-Chem-Plus-AP-Kit, Zytomed, Berlin, Germany); washing with Tris-HCl buffer; 20 min incubation with Streptavidin-AP-Conjugate (Zyto-Chem-Plus-AP-Kit, Zytomed, Berlin, Germany); washing with Tris-HCl buffer; staining with Fast Red (F4648, Sigma, Bad Homburg, Germany) twice for 5 min; washing with Tris-HCl buffer. Hematoxylin (S 3301, DAKO, Hamburg, Germany) was used as counterstaining. The histopathological examination was done by Dr. Gudrun Wibbelt (Leibniz Institute for Zoo and Wildlife Research [IZW], Berlin, Germany).

Sample collection, RT-PCR and CDV sequence analysis

This outbreak occurred during the period I spent with field work in the Serengeti and I did the necropsy and collected samples from two of the dead pups. The additional samples were collected and provided by Tanzanian colleagues (Dr. Robert Fyumagwa, Tanzania Wildlife Research Institute, Arusha, Tanzania; Dr. Morris Kilewo, Tanzania Nationalparks Authority, Arusha, Tanzania). Tissue samples were processed for RT-PCR and phylogenetic analyses as described in Chapter 2. I performed RT - PCRs using *morbillivirus*-specific primers that amplify a 388 nt fragment of the P gene and a 335 nt fragment of the F gene (Table 18). I generated neighbour-joining trees of the P and F gene fragments using the Tamura Nei parameter with 1000 bootstrap pseudo-replications as implemented in Mega 4 (Tamura et al. 2007).

When identical nt sequences for the fragments were available from a particular host species, only one sequence was used in the analysis. Geographical origin, host species and GenBank accession numbers of variants included in the phylogenetic analyses are indicated in the phylogenetic trees (Fig. 18). Nt sequence data for variant SNP_hyena_94-177 see Carpenter et al. (1998).

Detection of concurrent infections

Screenings for CDV, CPV, *Hepatozoon* sp. and *Babesia* sp. were conducted at the IZW. The primer pairs used and publications detailing the methods applied for pathogen screening are described in Table 18. PCRs were replicated at least twice and products confirmed by sequencing. The presence of *Streptococcus equi* was determined by cultivation of bacteria and molecular methods as described by Höner et al. (2006); bacterial investigation was performed by Dr. Stephanie Speck (IZW). Tissues were screened for the presence of *Babesia* sp. using specific primers (Table 18) by Veljko Nikolin and Martina Matzke (IZW). Rabies screening was carried out at the Friedrich-Loeffler-Institute, Federal Research Centre for Animal Health, Wusterhausen/Dosse, Germany, by Dr. Thomas Müller. Brain samples from six animals were preserved in phosphate buffered 50% glycerol solution and screened for rabies virus. Rabies virus antigen in brain smears was detected using the standard fluorescent antibody test (FAT) (Dean et al. 1996) and confirmed by a quantitative real-time PCR (qRT-PCR) targeting the nucleoprotein (N) gene of rabies virus essentially as described (Wakeley et al. 2005).

Table 18: Primers and target genes used for pathogen screening, plus amplicon size from target genes.

Pathogen	Primer	Sequence (5' → 3')	Target gene	Amplicon size [nt]	Reference
CDV	P1	ATGTTTATGATCACAGCGGT	P	429	Barrett et al. 1993
	P2	ATTGGGTTGCACCACTTGTC			
CDV	FC1	GGACTGATAATGTCCATTA	F	372	Liermann et al. 1998
	FC2	ATAGCTTTGTTAGACTGTT			
CPV	555for	CAGGAAGATATCCAGAAGGA	VP2	583	Buonavoglia et al. 2001
	555rev	GGTGCTAGTTGATATGTAATAAACA			
Rabies virus	JW12 F	ATGTAACACCYCTACAATG	N	110	Wakeley et al. 2005
	N165–146R	GCAGGGTAYTTRTACTCATA			
	LysGT1 P	ACAAGATTGTATTCAAAGTCAATAATCAG			
<i>Streptococcus equi</i> subsp. <i>ruminantium</i>	TPU1	AGAGTTTGATCMTGGCTCAG	16S	1369	Wyss et al. 1996
	RTU8	AAGGAGGTGATCCAKCCRCA	rRNA		
	Strep2F	TAACTAACCAGAAAGGGACG	Sequencing primers		Höner et al. 2006
	Strep3R	CACGAGCTGACGACAACC			
	Strep4F	AGAACCTTACCAGGTCTTGAC			
	Strep5R	TCAGACTTATTAACCGCCTG			
<i>Hepatozoon</i> sp.	Hemo3	TGTGTACAAAGGGCAGGGACG	18S	462	East et al. 2008
	Hemo4	GCGGCTTAATTTGACTCAACAC	rRNA		
<i>Babesia</i> sp.	Babspec for	CCGTGSTAATTSTAGGGCTAATAC	18S	687	this study
	Babspec rev	AACCAAAGTCCTACTCTATTATTCATG	rRNA		

3.6.3 RESULTS

Disease outbreak

On 3rd October 2007, 23 (nine adults, six yearlings and eight pups) of approximately 38 members of one pack of African wild dogs (the Ololosokwan pack) were found dead or dying during a period of eight days in the Loliondo Game Controlled Area (Fig. 16). Affected animals showed clinical signs of ataxia, weakness, soiling of the perineum and dehydration. Some carcasses were markedly decomposed.

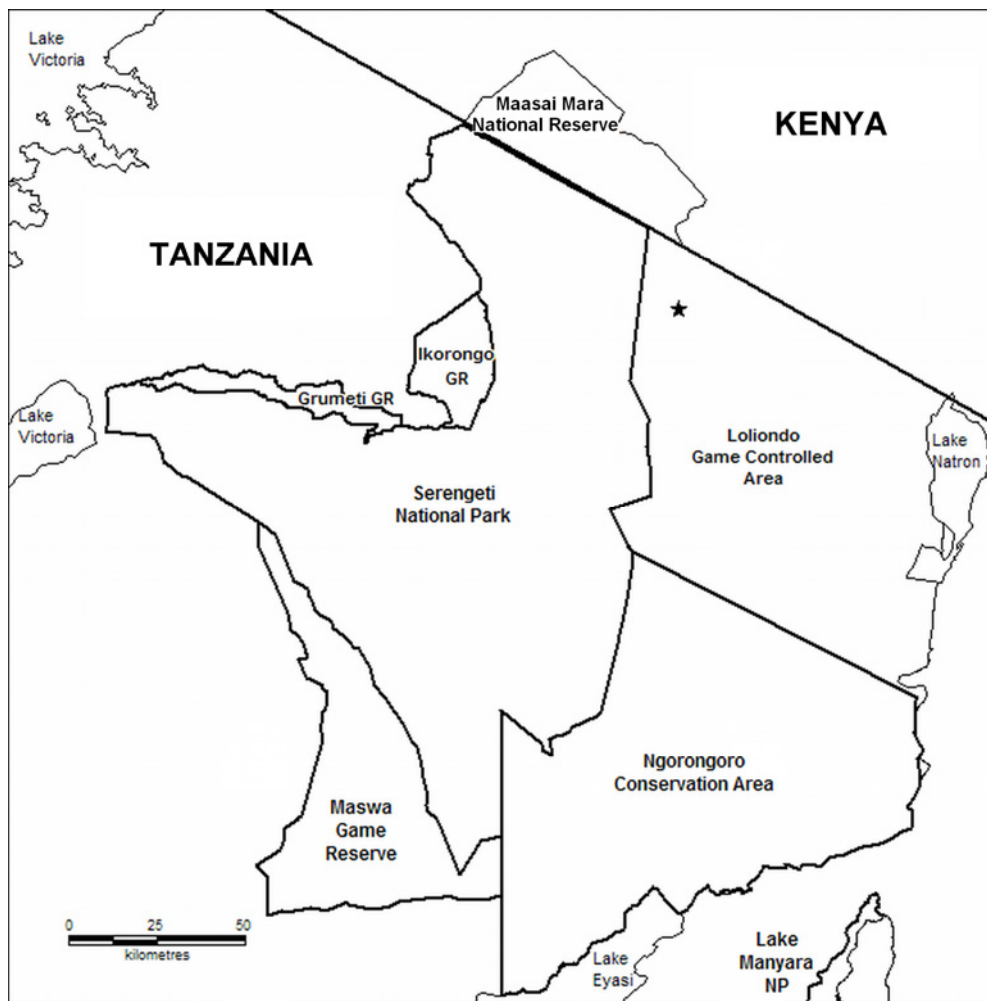


Figure 16: Map of the Serengeti National Park, Ngorongoro Conservation Area and Loliondo Game Controlled Area in Tanzania and the Maasai Mara National Reserve, Kenya. The location of the African wild dog pack infected with canine distemper virus is marked with a black star (GR, Game Reserve; NP, National Park).

Histopathology

Histopathological examination of the organs from six animals (four pups and two adults) identified that the main pathological changes were located in the lungs (Table 19). Moderate to severe multilobular suppurative to necrotizing bronchopneumonia was found with extensive intra-alveolar and interstitial infiltration with mononuclear inflammatory cells and some

neutrophilic granulocytes, as well as marked (secondary) bacterial colonisation. Epithelial lining cells of bronchi and bronchioli contained clearly visible eosinophilic intracytoplasmic inclusion bodies (Fig. 17a). Immunohistochemistry revealed that respiratory epithelial cells and their inclusion bodies reacted clearly positive with antibodies against CDV antigen (Fig. 17b). Additionally, a few animals had formation of multiple syncytial cells within the parenchymal tissue (Fig. 17c), some also reacting positive with antibodies against CDV antigen. The severity of the findings is consistent with fatal canine distemper virus infection. Further pathological changes of other organs were considered incidental findings and were mostly confined to generalised congestion.

Table 19: Summary of pathological changes found in different organs from six African wild dogs. Individuals 1-4 were pups (< 12 months of age); individuals 5-6 were adults.

Organs and pathological changes	1	2	3	4	5	6
Lung: Bronchopneumonia	+++*	+++*	+++*	++*	+++*	+++*
Lung: Syncytial cells	-	-	-	++*	++*	-
Lung: Interstitial oedema	+++	++	++	-	++	+++
Lung: Intra-alveolar haemorrhage	-	+	+	-	++	++
Respiratory epithelium: Intracytoplasmic inclusion bodies	+++*	+++*	++*	++*	+++*	+++*
Small intestine:	-	++	nd	+	+	+++
Coccidial infection						
Spleen:	++	++	nd	++	nd	nd
Depletion of lymph follicles						
Heart: Focal myocarditis associated with protozoal cyst	nd	+	nd	nd	nd	nd
Lymph nodes:	nd	++	nd	nd	nd	nd
Follicular hyperplasia						
Liver	NAD	NAD	NAD	NAD	NAD	NAD
Kidney	NAD	NAD	NAD	NAD	NAD	NAD
Brain	NAD	nd	nd	nd	nd	nd
Degree of autolysis	-	-	++	+++	+	+

-: none; +: mild; ++: moderate; +++: severe histological changes; *containing cells positive for canine distemper virus antigen; NAD: nothing abnormal detected; nd: not determined

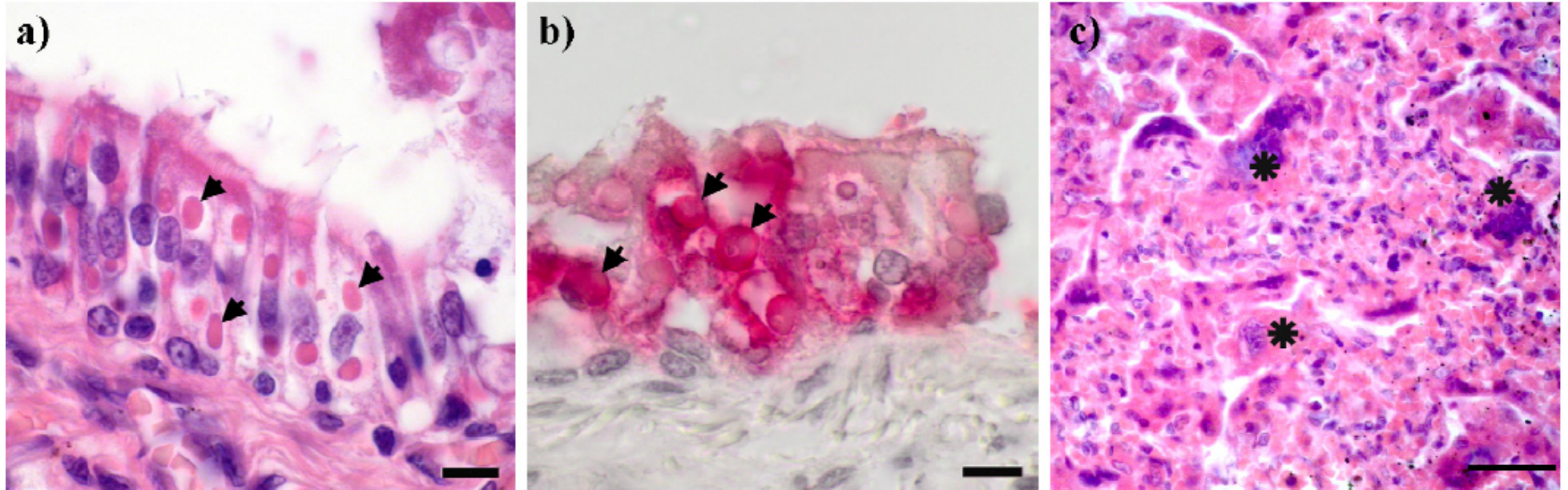


Figure 17: CDV related pathological changes in lung tissues of infected animals.

(a) Bronchiolar epithelium with multiple eosinophilic viral intracytoplasmic inclusions bodies (arrows) (bar = 10µm). (b) Positive immunohistochemistry reaction of bronchiolar epithelial cells and inclusion bodies (arrows) against canine distemper virus antigen (bar = 10µm). (c) Lung tissue, multiple syncytial cells (asterisks) (bar = 50µm).

RT-PCR and CDV P and F gene fragment sequences

I submitted the CDV sequences I generated in this study to GenBank. Two sequences were obtained for a 388 nt fragment of the P gene (GenBank accession numbers: EU481827, EU481828) and phylogenetic comparison of sequence data from these fragments with published information (Fig. 18a) revealed that the African wild dog pack in 2007 was infected with a CDV variant most closely related to a CDV variant described from a domestic dog (100% identity) in the Ngorongoro Conservation Area in 1994 and closely related to a variant described in a captive African wild dog (99% similarity) in north-eastern Tanzania in 2000, and to CDV variants previously described from lions, a spotted hyena, and a bat-eared fox in the Serengeti National Park in northern Tanzania in 1993 and 1994. Only one other P gene fragment from Africa is available which is from a domestic dog in Namibia. All P gene fragments from African CDV variants clustered together with the Tanzania variants forming a distinct and tight sub-cluster. Similarly, the 335 nt fragment of the F gene from one pup (GenBank accession number: EU481829) was most closely related (99% similarity) to the variant that infected a spotted hyena during the CDV outbreak among wild carnivores in the Serengeti National Park in 1993 and 1994 (AF026233), the only available F gene fragment from Tanzania (Fig. 18b).

Concurrent infections

The results of molecular screening for concurrent infections in each of the six animals examined are summarized in Table 20. Of two pups tested for CPV infection, both yielded positive results. One of these two pups was also positive for *Streptococcus equi* subsp. *ruminantium* infection. All six pack members screened for *Hepatozoon* sp. infection were positive. None of the six pack members were positive for rabies or *Babesia* sp..

Table 20: Concurrent pathogens found infecting six African wild dogs from one pack. The organs listed were screened using various methods for viruses, two tick-borne blood parasites and one bacterium. Individuals 1-4 were pups (< 12 months of age); individuals 5-6 were adults.

Method	Organ	1	2	3	4	5	6
CDV RT-PCR	brain, lung	+	+	nd	nd	nd	nd
CPV PCR	lung, intestine	+	+	nd	nd	nd	nd
Rabies virus qRT-PCR	brain	-	-	-	-	-	-
<i>Streptococcus equi</i> subsp. culture PCR	lung	+	-	nd	nd	nd	nd
<i>Hepatozoon</i> sp. PCR	liver, kidney	+	+	+	+	+	+
<i>Babesia</i> sp. PCR	lung	-	-	-	-	-	-

-: negative; +: positive; nd: not determined.

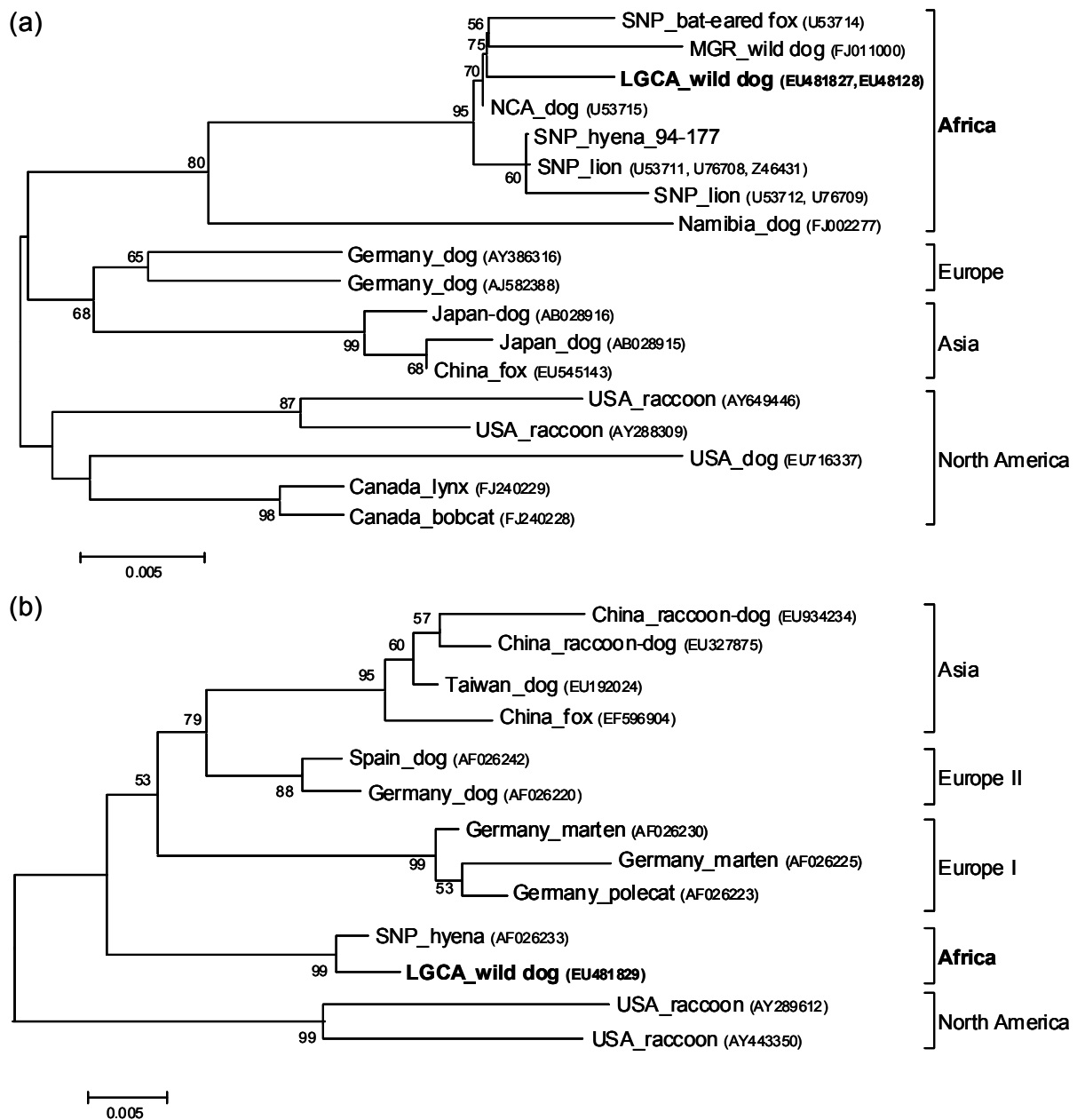


Figure 18: Phylogenetic relationship of the CDV variant from an African wild dog pack in the Loliondo Game Controlled Area, northern Tanzania to published variants from Tanzania and elsewhere. (a) Neighbour-joining tree based on a 388 nt fragment of the P gene. (b) Neighbour-joining tree based on a 355 nt fragment of the F gene generated.

3.6.4 DISCUSSION

Our histopathological results (Fig. 17) from six members of one African wild dog pack in the Serengeti ecosystem in 2007 indicate fatal CDV infection. I describe for the first time a CDV variant from a free-ranging pack using fragments of the P gene and F gene (Fig. 18). Phylogenetic analysis of the P gene fragment revealed high homology between the CDV variant that infected the pack in 2007 and other CDV variants previously described from Tanzania, including variants from fatal CDV infection of captive African wild dogs in the Mkomazi

Game Reserve in 2000 (van de Bildt et al. 2002) (now upgraded to a National Park) and from a CDV epidemic in wild carnivore hosts (lions, spotted hyenas and a bat-eared fox) in the Serengeti National Park, and a domestic dog in the Ngorongoro Conservation Area (Fig. 16) in 1993 and 1994 (Haas et al. 1996; Roelke-Parker et al. 1996; Carpenter et al. 1998). I also found high homology between one CDV F gene fragment from the pack in 2007 and one variant from a spotted hyena host in the Serengeti National Park in 1994 (Liermann et al. 1998). Evidence for one or more concurrent infections with CPV, *Hepatozoon* sp. and *Streptococcus equi* subsp. *ruminantium* was also found in the pack members examined. These concurrent infections may have occurred as a consequence of the immunological suppression caused by CDV infection (von Messling et al. 2004). It is also possible that some pack members were already infected with one or more pathogens when first exposed to CDV, and these infections, particularly CPV (Parrish & Kawaoka 2005), may have increased susceptibility to and the pathological consequences of CDV infection, irrespective of the sequence in which concurrent infections were acquired (Beldomenico et al. 2008). Thus our results indicate that the primary cause of morbidity and mortality in the pack was CDV disease and that co-infections may have contributed to the fatal outcome of CDV infection in some animals. Although all previous clinical diagnoses of African wild dog carcasses in both the Tanzanian and Kenyan sector of the Serengeti ecosystem have been positive for rabies infection (Burrows et al. 1994; East & Burrows 2001; Woodroffe 2001) none of the animals screened in this study were positive for rabies. Sera from lions in the Serengeti National Park provide evidence of increased CDV exposure in 2006 without infection causing disease in this host species (Munson et al. 2008). I am unaware of any evidence of clinical signs of CDV infection in any wild carnivore host species inside the park in either 2006 or 2007, even though several host species were closely monitored by researchers.

Although CDV and related viruses in the genus *Morbillivirus* are associated with disease epidemics in wildlife (Taubenberger et al. 1996; Harder & Osterhaus 1997; Akineden et al. 2005), current knowledge of the impact of CDV on the population dynamics of African wild dogs is largely unknown. Serological surveys indicate relatively high exposure to CDV in some African wild dog populations without apparent evidence of fatal disease, for example, in the Okavango Delta, Botswana (Alexander et al. 2010), and the Selous Game Reserve, Tanzania (Creel et al. 1997). Both Creel et al. (1997) and Alexander et al. (2010) suggest that African wild dog populations can remain demographically healthy despite high exposure to CDV. There is also evidence that African wild dogs on the Maasai steppe, Tanzania (Visser 2001), and in Namibia (Laurenson et al. 1997) are exposed to CDV. Although no evidence of

exposure to CDV was found in packs in the Kruger National Park, South Africa in the 1990s (van Heerden et al. 1995), a more recent study has revealed evidence of exposure (Cronwright-Snoeren 2010).

I know of only two previous clinically confirmed outbreaks of CDV infections in free-ranging African wild dog packs. CDV infection was established in one pack in Chobe National Park, Botswana in 1994 using immunohistochemistry on lung sections from one juvenile, however no genetic sequence data were obtained from the CDV variant and the pack of 12 animals was reduced to two surviving females following the death of six juveniles and four males (Alexander et al. 1996). The second possible CDV infected pack occurred in 1968 in the Serengeti ecosystem when five adults and 11 pups disappeared from a pack during a period of weeks leaving five adults and five pups, and one pup post mortem revealed haemorrhagic gastritis and enteritis, and heavy blood loss (Schaller 1972). Although these clinical signs are typical of the gastro-intestinal form of distemper they are not exclusive to CDV infection. The most extensive mortality reported in African wild dogs associated with CDV infection occurred in 2000 in fenced breeding enclosures in the Mkomazi Game Reserve, Tanzania, when 49 of 52 captive animals, most of which had been vaccinated against CDV (Visee 2001), died (van de Bildt et al. 2002). Interestingly, nine of 12 wild caught pups obtained from the Maasai steppe to start this captive breeding facility had positive CDV titres before they were vaccinated (Visee 2001).

There is evidence suggesting that CDV infection has occurred in wild carnivore hosts in the Serengeti ecosystem for the past few decades and not always with fatal consequences. A distemper-like disease reduced silver-backed jackal numbers between 1978 and 1979 (Moehlman 1983) without affecting sympatric golden jackals *Canis aureus* or African wild dog packs (Burrows et al. 1994). Serological evidence indicates CDV exposure among wild carnivores for many years before the start of the CDV epidemic in 1993, and high seroprevalence in 2006 occurred without evidence of clinical disease in infected species (Harrison et al. 2004; Munson et al. 2008).

Why one African wild dog pack close to the Serengeti National Park boundary (Fig. 16) in 2007 suffered fatal CDV infection when other wild carnivore host species in the park, and other African wild dog packs in the Loliondo Game Controlled Area displayed no clinical signs of CDV infection or increased mortality in that year, is unclear. It is possible that the immune status of the pack may have been compromised by intrinsic and extrinsic factors (Hofer & East 1998; Lee 2006; Beldomenico et al. 2008; Munson et al. 2008) that resulted in CDV infection causing fatalities among pack members. High contact rates between pack

members would ensure rapid transmission of infectious pathogens to all susceptible pack members, and infections that reduced pack hunting success would be expected to accelerate immunological decline (Beldomenico et al. 2008).

Despite the fact that this Chapter reports on confirmed fatal CDV infection in free-ranging African wild dogs, it is currently unclear what the demographic consequences of CDV are for African wild dog populations and why some packs survive exposure to CDV while others succumb to fatal infection. The level of natural herd immunity to CDV within a population is likely to be one key factor determining the impact of CDV (Guiserix et al. 2007), another may be the effect of concurrent infections on immune status. Although CDV infection can be fatal to African wild dogs, there is currently little evidence that this virus threatens large demographically healthy populations. Even so, high CDV mediated mortality within one or a few packs could result in a significant population reduction in small, isolated populations of this endangered canid.

4 GENERAL DISCUSSION

Little is known about the ecological role of wildlife pathogens for the long-term dynamics of wildlife host populations (Grenfell & Dobson 1995), the impact of non-virulent pathogens on life-history parameters of individuals (Lee 2006; Jones et al. 2008; Barrett et al. 2008) and the impact of simultaneous exposure to several pathogens on individual health.

The effects of various intrinsic and extrinsic factors on an individual's ability to respond to infections and potential interactions between these factors have been mainly studied in laboratory model species such as rodents and in humans. Much is also known about the impact of different variants of pathogens which infect livestock and companion animals. Far less is known about pathogens and their variants which infect wildlife populations and the consequences of such infection on pathogen evolution. With this general lack of knowledge in mind, my aim was to contribute to the knowledge on pathogen-host dynamics in a natural ecosystem where processes of natural selection still operate.

Basic aims

The basic aims of this study were to (1) develop non-invasive methods to identify pathogens in spotted hyenas and sympatric carnivores in the Serengeti ecosystem, Tanzania; (2) genetically determine which variants of several viruses and one tick-borne pathogen infected spotted hyenas and other sympatric carnivores in the Serengeti and further investigate the phylogenetic relationships between variants in different Serengeti carnivore species and those from other geographical regions; (3) determine infection patterns and persistence of these pathogens in two subpopulations of a spotted hyena metapopulation in this ecosystem; (4) investigate which life-history, social and ecological factors influence susceptibility to single and concurrent infections and study their impact on a key life-history parameter, the survival and longevity of spotted hyenas with known infection status during their juvenile stage. As during my field period in the Serengeti in 2007 a pack of African wild dogs died of fatal CDV infection in the Loliondo Game Controlled Area, close to the Serengeti National Park border, I extended my basic aims to additionally investigate why CDV, which significantly increased cub mortality in Serengeti spotted hyenas in 1993 and 1994, was not observed in spotted hyenas in 2007 but proved to be fatal to a pack of African wild dogs during that outbreak of infection in 2007.

Of key importance to my research was the fact that it was embedded in a long-term research programme that provided detailed life-history data on several hundred individually known

animals in two subpopulations (Hofer & East 1993 a, b; Höner et al. 2005). In social species such as the spotted hyena and some old world primates living in large social groups, research is often limited to one key study group for practical reasons. This can be a problem if the study group is not a “typical” group representative for the entire population. This was not a concern in my study as my results are based on many individuals from several social groups in each subpopulation which represents a substantial sample for a large carnivore species. As a result, the sample sizes in my statistical tests were likely to have sufficient statistical power.

Methodological approach

Pathogens that infect wildlife species may be genetically distinct from those described from domestic animals or humans and thus may be difficult to detect using standard primers (Si et al. 2010). As most pathogen variants circulating in the carnivore guild in the Serengeti ecosystem differed from those described and published in GenBank, I needed to establish methods that would reliably detect infection in the samples I screened. As my work was based on carnivores living in world heritage sites of international importance for biological conservation I had to develop methods to detect infection without resorting to invasive techniques. I therefore developed molecular biological methods for the detection of viral nucleic acids in faeces which could be non-invasively obtained from known individuals. This approach also permitted the collection of a far larger number of samples for screening than could have been obtained using the traditional approach of chemical immobilisation followed by sample collection. Equally important, within the context of the on-going long-term ecological and behavioural research, was the requirement to obtain samples from individuals without destroying habituation or incurring the potentially negative effects of immobilisations for handled animals such as death (Arnemo et al. 2006), reduced fecundity (Alibhai et al. 2001) and loss of body condition, altered behaviour or elevated physiological ‘stress’ (Cattet et al. 2008).

Spotted hyenas are more closely related to felids than canids but, as members of an ecological guild with both canid and felid species, they are likely to be infected with pathogens that come from feline or canine hosts (East et al. 2002, 2004, 2008). To establish methods for the detection of some of the viruses and a tick-borne pathogen I initially used published primer pairs to detect feline- and canine-like pathogen variants and designed novel specific primer pairs to screen for hyena variants (Chapters 3.2 and 3.5).

Phylogeny and pathogen transmission

My phylogenetic studies revealed several novel results. These particularly included an unexpectedly high diversity of coronavirus variants and a complete turnover from feline to canine

variants infecting my study population within a few years. This may have resulted from rapid evolution of coronavirus in the spotted hyena host or the recent spread of a successful variant from another host species (Chapter 3.1) – the current data do not permit a clear preference for either hypothesis. These results highlight the need for more long-term monitoring of genetic variants of viruses such as coronavirus in wildlife host populations to obtain a better understanding of what factors determine variant diversity and evolution, and variant transmission within and between host populations.

Although spotted hyenas were infected by many genetic variants of typical ‘domestic cat’ or ‘domestic dog’ pathogens, my results on calicivirus infection revealed variants more closely related to human sapoviruses than to feline or canine caliciviruses. I speculate that this unusual host-pathogen association may have been established through the consumption of human carcasses or contaminated human waste by spotted hyenas, suggesting a host species jump from human caliciviruses to spotted hyenas (Chapter 3.2).

My phylogenetic investigation of the tick-borne apicomplexan protozoan *Hepatozoon* revealed that several undescribed novel *Hepatozoon* species infected the guild of carnivores in the Serengeti, with a high prevalence of infection in spotted hyenas, lions and African wild dogs and a rather low one in bat-eared foxes, probably because transmission is a consequence of ingestion of prey-associated ticks. Spotted hyenas and sympatric carnivores in the Serengeti National Park and the Ngorongoro Crater were infected with at least two *Hepatozoon* species which are closely related and were distinct from another novel *Hepatozoon* species which infected African wild dogs in the Loliondo Game Controlled Area outside the National park. I speculate that this particular difference resulted from prey-associated ingestion of ticks from livestock in Loliondo Game Controlled Area and wild herbivores within the Serengeti ecosystem (Chapter 3.5).

Phylogenetic analysis of the CDV variant responsible for the death of a pack of the endangered African wild dog close to the border of the Serengeti National Park in 2007 revealed a high homology to CDV variants previously described from Tanzania, including variants from fatal CDV infection in captive African wild dogs in the Mkomazi Game Reserve in 2000 (van de Bildt et al. 2002) and a CDV epidemic in wild carnivores in the Serengeti National Park and a domestic dog in the Ngorongoro Conservation Area in 1993 and 1994 (Haas et al. 1996; Roelke-Parker et al. 1996; Carpenter et al. 1998). It is unclear why this variant was fatal for African wild dogs in 2007. I suggest that co-infection with *Hepatozoon* and canine parvovirus may have contributed to the severity of clinical symptoms since there was no evidence of a spread of this variant to other wildlife species in the Serengeti National Park (Chapter 3.6).

Patterns of persistence of non-virulent viral infections

In social mammal populations, the ability of a pathogen to persist depends on factors which include pathogen virulence, transmission properties of the pathogen, host social structure, host age, and the immunocompetence of individual hosts (Anderson & May 1991; Lindholm & Britton 2007) which in turn may be influenced by several life history parameters (Chapter 3.3). Using coronavirus as an example of a widely spread, non-virulent pathogen in my study population, I investigated prevalence of infection in two subpopulations of a metapopulation that differ in ecological and demographic traits, the spotted hyenas in the Serengeti National Park and the adjacent Ngorongoro Crater. Although I found serological evidence of coronavirus infection in both subpopulations, I only detected active virus excretion in the Serengeti subpopulation. I used the pattern of coronavirus infection across age classes within the Serengeti subpopulation to look at viral transmission and persistence. My results suggest that a key difference between the Serengeti and Crater subpopulation was the substantial difference in group size and temporal availability of susceptible juveniles which – probably through demographic stochasticity – led to unfavourable conditions in the Crater but favourable conditions of persistence in the Serengeti and indicate that coronavirus recently went extinct in the Crater subpopulation (Chapter 3.4).

Effects of life-history traits and environmental parameters on infection status

Theoretical models consider how life-history traits, social processes and environmental factors structure interactions and thus influence pathogen transmission in social species (e.g. Bonds et al. 2005; Christley et al. 2005). I used this body of knowledge on the interplay of social factors such as social status and dominance status with nutritional status and age to derive predictions for my analysis and develop the statistical models that establish which factors were likely to influence infection status of juveniles and their subsequent longevity (Chapter 3.3).

Young animals with a naïve immune system are typically more susceptible to infection and may suffer more severely from infections than older animals. As expected, cubs were more likely to acquire infection with coronavirus (Chapter 3.1) and calicivirus (Chapter 3.2 and 3.3) than older individuals, and infection with the tick-borne pathogen *Hepatozoon* caused mortality in cubs (East et al. 2004, 2008, Chapter 3.5) although it was apparently benign in older animals.

Pathogen transmission often depends on the contact rate between infected and susceptible individuals within and between populations or social groups (Lindholm & Britton 2007;

Webb et al. 2007). Social environmental effects that result from dominance structures within social groups may determine access to key resources such as food. The dominance hierarchy in spotted hyenas mediates access to resources within a clan territory (Frank 1986) and thereby influences the ability of animals of different rank to allocate body resources to growth (Hofer & East 2003) and reproduction (Holekamp et al. 1996; Hofer & East 2003), and thus most likely also determines the ability of animals to invest in body maintenance which includes immune function. Within-litter dominance relationships also strongly influence access of littermates to maternal teats (Hofer & East 1997; Wachter et al. 2002) and consequently growth of littermates (Hofer & East 2008), and therefore probably the ability of dominant and subordinate cubs to allocate body resources to immune function. Members of twin litters were more susceptible to infection with coronavirus or calicivirus than singletons, and especially subordinate cubs of twin litters had an increased likelihood to be infected with calicivirus, probably as a result of reduced nutritional status and social as well as physiological ‘stress’ (Chapter 3.3). During times of low prey abundance, likelihood of infection was high, suggesting that juveniles suffered from nutritional ‘stress’ and reduced immune function. Interestingly, the likelihood of infection was also high during times of high prey abundance, probably the consequence of higher contact rates between infected and susceptible individuals (Chapter 3.3).

In hosts simultaneously infected with more than one pathogen, either micro-parasites or macro-parasites, pathogens may compete for host resources or be responsible for contradictory immune responses, and such interactions may affect the fitness and virulence of the pathogens involved. Infection with one pathogen might result in reduced immune function and thus could lead to an increased susceptibility of the host to infection with further pathogens (Graham et al. 2007; Cattadori et al. 2008). Unexpectedly, cubs infected with a macro-parasite (helminths) were less likely to be infected with coronavirus or co-infected with both coronavirus and calicivirus. I suggest this phenomenon may be a result of helminth-induced local immune responses in the gastrointestinal tract that undermine the ability of these enteric viruses to infect their host (Chapter 3.3).

Impact on survival and longevity

The impact of virulent pathogens on host survival is obvious. The idea that pathogens of low virulence may influence life history parameters is less obvious and rarely considered. The fact that concurrent infection with pathogens of low virulence may lead to synergistic effects that influence life history parameters is known from experimental studies on laboratory species and humans, but there are few studies to have investigated the impact of concurrent infections

in free-ranging wildlife (Cattadori et al. 2007). In my study I investigated the potential impact of pathogens with low virulence on the longevity of individuals whose infection status was assessed as juveniles. I constructed a statistical model to consider single pathogens or the potential synergistic reaction of concurrent infection of two enteric viruses, coronavirus and calicivirus, and demonstrated that non-virulent pathogen infection with coronavirus in juveniles can indeed increase the cumulative hazard and thus reduce longevity (Chapter 3.3).

Canine distemper virus outbreak

Finally I demonstrate the troubling presence of CDV in a pack of African wild dogs in a year in which I found no evidence for CDV infection in my study population of spotted hyenas. The genetic type of this CDV variant was not sufficiently different to suggest a lack of pathogenicity in spotted hyenas. Examination of virulent CDV infection in one pack does not permit me to draw strong conclusions but I suggest that the concurrent infections that I detected in the pack members that I screened provide some evidence that the immune status of the pack may have been compromised by these concurrent infections, particularly parvovirus, and that these may explain why only this pack died (Goller et al. 2010; Chapter 3.6). This idea also suggests that for some reason, the general level of immunity among spotted hyenas, and particularly in young cubs, in the Serengeti National Park in 1993 and 1994 was reduced compared to other years, thereby increasing the pathological effect of CDV infection on young animals. It was suggested that this was the case in the lion population because of high levels of *Babesia* sp. infection (Munson et al. 2008). However, high *Babesia* infection is unlikely to explain the occurrence of clinical CDV in spotted hyenas in 1993, as there is now evidence that lions and spotted hyenas were infected with different *Babesia* species (Matzke 2010) and unlike the lion population in which animals of all age classes died, clinical CDV was only apparent in young spotted hyenas (Haas et al. 1996).

Closing remarks

Results presented in this thesis highlight the value of using the theoretical background and analytical tools developed in several different biological disciplines to answer some key questions concerning pathogen-host interactions in wildlife host populations that have received little attention. Several novel results produced in my study make a substantial contribution to the currently limited knowledge of pathogen-carnivore host dynamics in African savannah ecosystems. My study considered relatively few of the probably numerous pathogens maintained in the numerous carnivore host species in the Serengeti ecosystem and without doubt there are still many pathogens waiting to be described. My genetic techniques only considered

relatively short fragments of particular genes. New developments in genetic techniques will soon make it possible to consider far more extensive sequence data from the genomes of pathogens and hosts, and this will extend our understanding of the molecular basis of virus-host specificity and co-evolution beyond what was possible in my current study.

My study was focused on the spotted hyena which is only one of many carnivore host species in the Serengeti. To obtain a deeper insight into whether a particular pathogen or variant infects one or more host species and which factors determine host range, large-scale screening of samples from sympatric host species is needed. Currently there is little known about the presence or absence of receptors used by pathogens to gain entry into host cells in most African carnivores, including the spotted hyena. Even so, although host range may be determined by the presence or absence of host entry mechanisms such as receptors, it is also possible that the host range of any variant may be influenced by transmission dynamics such as the likelihood of a variant being transmitted from one host species to another. For infectious diseases, more needs to be known about inter-specific contact, and for tick-borne diseases more information is required about vector species and the variants they transmit. Finally, long-term monitoring of host populations and an assessment of the life-history consequences of pathogen infection in wild carnivores is essential if we are to obtain an understanding of pathogen-host dynamics in wildlife and how these dynamics evolve over time.

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ABBREVIATIONS

A	average age at infection
aa	amino acid
AIDS	acquired immunodeficiency syndrome
AWD	African wild dog
CaCV	canine calicivirus
CDV	canine distemper virus
CPV	canine parvovirus
CRFK	Crandall feline kidney cells
CV	calicivirus
DEPC	Diethylpyrocarbonate
DNA	deoxyribonucleic acid
DWM	dwarf mongoose
EBHSV	European brown hare syndrome
FCV	feline calicivirus
FHV	feline herpesvirus
FIV	feline immunodeficiency virus
FPLV	feline panleukopenia virus
FX	bat eared fox
GALT	gut-associated lymphoid tissue
GE	common genet
HIV	human immunodeficiency virus
HY	hyena
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
JTT	Jones Taylor Thornton
L	life expectancy
LGCA	Loliondo Game Controlled Area
LI	lion
MCV	mink calicivirus
ML	maximum likelihood
MP	maximum parsimony
mt	mitochondrial
N	sample size
NC	Ngorongoro Crater
NJ	neighbour joining
NoV	norovirus
nt	nucleotide
ORF	open reading frame
PCR	polymerase chain reaction
PDV	phocine distemper virus
R_0	basic reproductive rate
RBC	red blood cells
RdRp	RNA dependant RNA polymerase
RHDV	rabbit hemorrhagic disease virus
RNA	ribo nucleic acid
RT-PCR	reverse transcriptase polymerase chain reaction
SaV	Sapovirus
SBJ	silver-backed jackal

SE	serval cat
SIV	simian immunodeficiency virus
SLM	slender mongoose
SMSV	San Miguel sea lion virus
SNP	Serengeti National Park
sp.	species
subsp.	subspecies
SSJ	side-striped jackal
TCID	tissue culture infectious dose
th	T helper cells
VESV	vesicular exanthema of swine
WBC	white blood cells
WTM	white-tailed mongoose
χ^2	chi square

Abbreviations of amino acids

A	alanine
C	cysteine
D	aspartic acid
E	glutamic acid
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
W	tryptophan
Y	tyrosine

APPENDIX

A. Blood and tissue samples screened from spotted hyenas and sympatric carnivores collected in the Serengeti National Park (SNP), the Ngorongoro Crater (NC) and the Loliondo Game Controlled Area (LGCA). (HY: spotted hyena; SBJ: silver-backed jackal; LI: lion; FX: bat-eared fox; CI: civet; DWM: dwarf mongoose; SE: serval cat, SLM: SSJ: side-striped jackal; WTM: white-tailed mongoose; AWD: African wild dog)

Organ	SNP/NC			SNP								LGCA	Total
	HY	SBJ	LI	FX	CI	DWM	GE	SE	SLM	SSJ	WTM	AWD	
lung	21/8	-	3/1	4	1	1	2	1	1	-	1	6	50
lymph node	6/4	1/-	1/-	3	-	-	-	-	-	1	-	-	16
muscle	3/-	1/-	2/-	4	-	-	-	1	-	-	1	-	12
liver	2/3	-/1	-	-	-	-	1	-	1	-	-	-	8
spleen	1/1	-	-	2	-	-	-	-	-	-	-	-	4
kidney	2/-	-	-	-	-	-	1	-	-	-	-	-	3
heart	2/-	-	-	-	-	-	-	-	-	-	-	-	2
intestine	5/-	-	-	-	-	-	-	-	-	-	-	-	5
pancreas	1/-	-	-	-	-	-	-	-	-	-	-	-	1
salivary gland	-/2	-	-	-	-	-	-	-	-	-	-	-	2
testes	1/-	-	-	-	-	-	-	-	-	-	-	-	1
thyroid gland	2/-	-	-	-	-	-	-	-	-	-	-	-	2
tonsil	1/1	-	-	-	-	-	-	-	-	-	-	-	2
spleen	-	-	-	-	-	-	-	-	-	-	1	-	1
blood	38/-	-	5/-	-	-	-	-	-	-	-	-	-	43
Total	85/19	2/1	11/1	13	1	1	4	2	2	1	3	6	152

B. Nucleotide sequences of coronaviral M and S gene fragments

M gene sequences

CoV Hyena 36/04

CTTTAGTGTGTCAGGTGCAATTGTTACATTTACACTTTGGATTATGTATTTTGTAGATCCATTCAGTTATACAGAAGGACTA
 AGTCTTGGTGGTCTTTCAACCCTGAACTAATGCAATTCTTTGCGTTAGTGCATTAGGAAGAAGCTATGTGCTTCCTCTTGAA
 GGTGTGCCAACTGGTGTCACTTTAACATTGCTCTCAGGGAATTTGTATGCTGAAGGGTTCAAAATTGCAGGTGGTATGAACAT
 CGACAATTTGCCAAAGTACGTAATGGTTGCATTACCTAGCAGAACCATTTGTCTACACACTTGTGGTAAGAACTGAAAGCAA
 GTAGTGCAACAGGATGGGCTTACTATGTAAAATCTAA

CoV Hyena 42/04_1

CTTTAGTGTGTCAGGTGTCAGTTGTAACATTTGCACTTTGGATGATGTATTTTGTGAGATCTATTCAGTTATATAGAAGGACCA
 AATCATGGTGGTCTTTTCAACCCTGAACTAATGCAATTCTTTGTGTTAACGCGTTGGGTAGAAGCTATGTTTTGCCCTTGAT
 GGCACACCCACGGGTGTTACTCTCAGCTACTTTTCAGGAAATCTATATGCTGAAGGTTTTAAATGGCTGGTGGTCTCACCAT

TGAACATTTACCTAAATACGTCATGATTGCTACGCCTAATAGAACCATCGTTTACACATTAGTTGGAAAACAATTGAAAGCAA
CTACTGCCACGGGATGGGCTTACTATGTAAAATCTAA

CoV Hyena 42/04_2 (M478 040122)

CTTTAGTGTTGCAGGTGCAATTGTTACATTTATACTTTGGATTATGTATTTTGTAGATCCATTCAGCTATATAGAAGGACTA
AGTCATGGTGGTCTTTCAACCCTGAAACTAATGCAATTCTTTGCGTTAGTGCATTAGGAAGAAGCTATGTGCTGCCTCTTGAA
GGAGTGCCAACTGGTGTCACTTTAACGTTACTGTCAGGGAATTTGTATGCTGAAGGGTTCAAAATTGCAGGTGGTATGAACAT
CGACAATTTGCCAAAGTACGTAATGGTTGCATTACCGAGCAGAACCATTGTCTACACATTTGTTGGTAAGAACTGAAAGCAA
GTAGTGCAACAGGATGGGCTTACTATGTAAAATCTAA

CoV Hyena 64/06 (I433 060624)

CTTTAGTGTTGCATGTGCAATTGTTACATTTATACTTTGGATTATGTATTTTGTAGATCCATTCAGTTATACAGAAGGACTA
AGTCTTGGTGGTCTTTCAACCCTGAAACTAACGCAATTCTTTGTGTTAGTGCATTAGGAAGAAGCTATGTGCTTCCTCTTGAA
GGTGTGCCAACTGGTGTCACTTTAACATTGCTCTCAGGGAATTTGTATGCTGAAGGGTTCAAAATTGCAGGTGGTATGAACAT
CGACAATTTGCCAAAGTACGTAATGGTAGCATTACCTAGCAGGACCATTGTCTACACACTTGTGGTAAGAAATTGAAAGCAA
GTAGTGCAACAGGATGGGCTTACTATGTAAAATCTAA

CoV Hyena 33/07 (CoV Hyenas 06/07)

CTTTAGTGTTGCATGTGCAATTGTTACATTTATACTTTGGATTATGTATTTTGTAGATCCATTCAGTTATACAGAAGGACTA
AGTCTTGGTGGTCTTTCAACCCTGAAACTAACGCAATTCTTTGTGTTAGTGCATTAGGAAGAAGCTATGTGCTTCCTCTTGAA
GGTGTGCCAACTGGTGTCACTTTAACATTGCTCTCAGGGAATTTGTATGCTGAAGGGTTCAAAATTGCAGGTGGTATGAACAT
CGACAATTTGCCAAAGTACGTAATGGTAGCATTACCTAGCAGGACCATTGTCTACACACTTGTGGTAAGAAATTGAAAGCAA
GTAGTGCAACAGGATGGGCTTACTATGTAAAATCTAA

CoV Hyena 34/07 (CoV Hyenas 06/07)

CTTTAGTGTTGCATGTGCAATTGTTACATTTATACTTTGGATTATGTATTTTGTAGATCCATTCAGTTATACAGAAGGACTA
AGTCTTGGTGGTCTTTCAACCCTGAAACTAACGCAATTCTTTGTGTTAGTGCATTAGGAAGAAGCTATGTGCTTCCTCTTGAA
GGTGTGCCAACTGGTGTCACTTTAACATTGCTCTCAGGGAATTTGTATGCTGAAGGGTTCAAAATTGCAGGTGGTATGAACAT
CGACAATTTGCCAAAGTACGTAATGGTAGCATTACCTAGCAGGACCATTGTCTACACACTTGTGGTAAGAAATTGAAAGCAA
GTAGTGCAACAGGATGGGCTTACTATGTAAAATCTAA

CoV Hyena 35/07 (CoV Hyenas 06/07)

cTtTAGTGTTGCATGtGCAATTGTTACATTTATACTTTGGATTATGTAtTtTGTTAGATCCATTCAGTTATACAGAAGGAcTA
AGTCTTGGTGGTCTTTCAACCCTGAAACTAACGCAATTCTTTGTGTTAGTGCATTAGGAAGAAGCTATGTGCTTCCTCTTGAA
GGTGTGCCAACTGGTGTCACTTTAACATTGCTCTCAGGGAATTTGTATGCTGAAGGGTTCAAAATTGCAGGTGGTATGAACAT
CGACAATTTGCCAAAGTACGTAATGGTAGCATTACCTAGCAGGACCATTGTCTACACACTTGTGGTAAGAAATTGAAAGCAA
GTAGTGCAACAGGATGGGCTTACTATGTAAAATCTAA

CoV Hyena 36/07 (CoV Hyenas 06/07)

cttTAGTGtGCATGtGCAATTGTTACATTTATACTTTGGATTATGTATTTTGTAGATCCATTCAGTTATACAGAAGGAcTA
AGTCTTGGTGGTCTTTCAACCCTGAAACTAACGCAATTCTTTGTGTTAGTGCATTAGGAAGAAGCTATGTGCTTCCTCTTGAA
GGTGTGCCAACTGGTGTCACTTTAACATTGCTCTCAGGGAATTTGTATGCTGAAGGGTTCAAAATTGCAGGTGGTATGAACAT
CGACAATTTGCCAAAGTACGTAATGGTAGCATTACCTAGCAGGACCATTGTCTACACACTTGTGGTAAGAAATTGAAAGCAA
GTAGTGCAACAGGATGGGCTTACTATGTAAAATCTAA

CoV Hyena 37/07 (CoV Hyenas 06/07)

CTTTAGTGTTGCATGTGCAATTGTTACATTTATACTTTGGATTATGTATTTTGTAGATCCATTCAGTTATACAGAAGGACTA
AGTCTTGGTGGTCTTTCAACCCTGAAACTAACGCAATTCTTTGTGTTAGTGCATTAGGAAGAAGCTATGTGCTTCCTCTTGAA

GGTGTGCCAACTGGTGTCACTTTAACATTGCTCTCAGGGAATTTGTATGCTGAAGGGTTCAAAATTGCAGGTGGTATGAACAT
CGACAATTTGCCAAAGTACGTAATGGTAGCATTACCTAGCAGGACCATTGTCTACACACTTGTTGGTAAGAAATTGAAAGCAA
GTAGTGCAACAGGATGGGCTTACTATGTAAAATCTAA

CoV Hyena 44/07 (CoV Hyenas 06/07)

CTTTAGTGTTGCATGTGCAATTGTTACATTTATACTTTGGATTATGTATTTTGTTAGATCCATTTCAGTTATACAGAAGGACTA
AGTCTTGGTGGTCTTTCAACCCTGAAACTAACGCAATTCTTTGTGTTAGTGCATTAGGAAGAAGCTATGTGCTTCCTCTTGAA
GGTGTGCCAACTGGTGTCACTTTAACATTGCTCTCAGGGAATTTGTATGCTGAAGGGTTCAAAATTGCAGGTGGTATGAACAT
CGACAATTTGCCAAAGTACGTAATGGTAGCATTACCTAGCAGGACCATTGTCTACACACTTGTTGGTAAGAAATTGAAAGCAA
GTAGTGCAACAGGATGGGCTTACTATGTAAAATCTAA

CoV Hyena 62/07 (CoV Hyenas 06/07)

CTTTAGTGTTGCATGTGCAATTGTTACATTTATACTTTGGATTATGTATTTTGTTAGATCCATTTCAGTTATACAGAAGGACTA
AGTCTTGGTGGTCTTTCAACCCTGAAACTAACGCAATTCTTTGTGTTAGTGCATTAGGAAGAAGCTATGTGCTTCCTCTTGAA
GGTGTGCCAACTGGTGTCACTTTAACATTGCTCTCAGGGAATTTGTATGCTGAAGGGTTCAAAATTGCAGGTGGTATGAACAT
CGACAATTTGCCAAAGTACGTAATGGTAGCATTACCTAGCAGGACCATTGTCTACACACTTGTTGGTAAGAAATTGAAAGCAA
GTAGTGCAACAGGATGGGCTTACTATGTAAAATCTAA

CoV Hyena 82/07 (CoV Hyenas 06/07)

CtTtAGTGTTGCATGTGCAATTGTTACATTTATACTTTGGATTATGTATTTTGTTAGATCCATTTCAGTTATACAGAAGGACTA
AGTCTTGGTGGTCTTTCAACCCTGAAACTAACGCAATTCTTTGTGTTAGTGCATTAGGAAGAAGCTATGTGCTTCCTCTTGAA
GGTGTGCCAACTGGTGTCACTTTAACATTGCTCTCAGGGAATTTGTATGCTGAAGGGTTCAAAATTGCAGGTGGTATGAACAT
CGACAATTTGCCAAAGTACGTAATGGTAGCATTACCTAGCAGGACCATTGTCTACACACTTGTTGGTAAGAAATTGAAAGCAA
GTAGTGCAACAGGATGGGCTTACTATGTAAAATCTAA

CoV Hyena 91/07

CTTTAGTGTTGCATGTGCAATTGTTACATTTATACTTTGGATTATGTATTTTGTTAGATCCATTTCAGTTATACAGAAGGACTA
AGTCTTGGTGGTCTTTCAACCCTGAAACTAACGCAATTCTTTGTGTTAGTGCATTAGGAAGAAGCTATGTGCTTCCTCTTGAA
GGTGTGCCAACTGGTGTCACTTTAACATTGCTCTCAGGGAATTTGTATGCTGAAGGGTTCAAAATTGCAGGTGGTATGAACAT
CGACAATTTGCCACAGTACGTAATGGTAGCATTACCTAGCAGGACCATTGTCTACACACTTGTTGGTAAGAAATTGAAAGCAA
GTAGTGCAACAGGATGGGCTTACTATGTAAAATCTAA

CoV Hyena 110/07 (CoV Hyenas 06/07)

CTTTAGTGTTGCATGTGCAATTGTTACATTTATACTTTGGATTATGTATTTTGTTAGATCCATTTCAGTTATACAGAAGGACTA
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GGTGTGCCAACTGGTGTCACTTTAACATTGCTCTCAGGGAATTTGTATGCTGAAGGGTTCAAAATTGCAGGTGGTATGAACAT
CGACAATTTGCCAAAGTACGTAATGGTAGCATTACCTAGCAGGACCATTGTCTACACACTTGTTGGTAAGAAATTGAAAGCAA
GTAGTGCAACAGGATGGGCTTACTATGTAAAATCTAA

CoV Jackal1/07

CTTTAGTGTTGCAGGTGCAATTGTTACATTTATACTTTGGATTATGTATTTTGTTAGATCCATTTCAGTTATACAGAAGGACTA
AGTCTTGGTGGTCTTTCAACCCTGAAACTAACGCAATTCTTTGCGTTAGTGCATTAGGAAGGAGCTATGTGCTTCCTCTTGAA
GGTGTGCCAACTGGTGTCACTTTAACATTGCTCTCAGGGAATTTGTACGCTGAAGGGTTCAAAATTGCAGGTGGTATGAACAT
CGACAATTTGCCAAAGTACGTAATGGTGCATTACCTAGCAGGACCATTGTCTACACACTTGTTGGTAAGAAATTGAAAGCAA
GTAGTGCAACAGGATGGGCTTACTATGTAAAATCTAA

S gene sequences

CoV Hyena 36/04

TAACCAGACAAGCAGAGGTTAGGGCTAGTAGACAACCTTGCTATGGACAAAGTTAATGAATGCGTTAGGTCCCAATCTCAGAGA
TTTGGATTCTGTGGTAATGGTACACATTTGTTTTCACTTGCAAATGCAGCACCAAATGGCATGATCTTCTTTCACACAGTACT
ATTACCAACAGCTTATGAAACTGTAAACAGCTTGGTCGGGTATTTGTGCTTCAGACGGCGATAGCACTTTTGGACTTGTCGTTA
AAGATGTTTCAGTTGACGTTATTTTCGTAATCTAGATGGCAAGTTCTATTTGACTCCCAGAACTATGTATCAGCCTAGAGCTGCA
ACTAGTTCAGATTTTGTTCAAATTGAAGGGTGTGATGTGTTGTTTGTCAATGCAACTGTAATTGACTTGCCCTAGTATTATACC
TGACTATATCGACATTAATCAGACTGTTCAAGACATATTAGAAAACCTACAGACCAAACCTGGACTGTACCTGAATTGACACTTG
ACATTTTTTAACGCAACCTATTTAAATCTGACTGGTGAAATTAATGACTTAGAATTCAGGTCAGAAAAGCTACATAACACCACG
GTCGAACTTGCTGTTCTCATTGACAATATTAACAATACATTAGTCAATCTTGAATGGCTCAATAGAA

CoV Hyena 42/04

TAACCAGACAAGCAGAGGTTAGGGCTAGTAGACAACCTTGCTAAGGACAAAGTTAATGAATGCGTTAGGTCCCAATCTCAGAGA
TTTGGATTCTGTGGTAATGGTACACATTTGTTTTCACTTGCAAATGCAGCACCAAATGGCATGATCTTCTTTCACACAGTACT
ATTACCAACAGCTTATGAAACTGTAAACAGCTTGGTCGGGTATTTGTGCTTCAGACGGCGCTAGCACTTTTGGACTTGTCGTTA
AAGATGTTTCAGTTGACGTTATTTTCGTAATCTAGATGGCAAGTTCTATTTGACTCCCAGAACTATGTATCAGCCTAGAGCTGCA
ACTAGTTCAGATTTTGTTCAAATTGAAGGGTGTGATGTGTTGTTTGTCAATGCAACTGTAATTGACTTGCCCTAGTATTATACC
TGACTATATCGACATTAATCAGACTGTTCAAGACATATTAGAAAACCTACAGACCAAACCTGGACTGTACCTGAATTGACACTTG
ACATTTTTTAACGCAACCTATTTAAATCTGACTGGTGAAATTAATGACTTAGAATTCAGGTCAGAAAAGCTACATAACACCACG
GTCGAACTTGCTGTTCTCATTGACAATATTAACAATACATTAGTCAATCTTGAATGGCTCAATAGAA

CoV Hyena 64/06

TAACCAGACTAGCAGAGGTTAGGGCTAGTAGACAACCTTGCTAAAGACAAGGTTAATGAATGCGTTAGGTCTCAATCCAGAGA
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ATTACCAACAGCTTATGAAACTGTGACGGCCTGGTCAGGTATTTGTGCGTCAGATGGCGATCGCACTTTTGGACTTGTTGTTA
AGGATGTTTCAGCTGACGCTATTTTCGCAATTTAGATGACAAATTCTACTTGACTCCTCGAACTATGTATCAGCCCAGAATTGCA
ACTAGTTCAGACTTTGTTTCAATAGAAAGTTGTGATGTGTTGTTTGTCAATGCAACTGTAATTGACTTGCCCTAGTATTATACC
TGACTATATTGATATTAATCAAACCTGTTTCAGGACATATTAGAAAATTTTAGACCAAATTGGACTGTACCTGAGTTGACACTTG
ATATTTTTTAACGCAACCTACTTAAACCTGACTGGTGAAATTAATGACTTAGAATTTAGGTCAGAGAAGCTACATAATACCACG
GTAGAACTTGCTGTTCTTATTGACAATATTAATAATACATTAATCAATCTTGAAT

CoV Hyena 33/07 (CoV Hyenas 07)

GTTAGTGCTAGTAGACAACCTTGCTAAAGACAAGGTTAATGAATGCGTTAGGTCTCAATCCAGAGATTTGGATTCTGTGGTAA
TGGTACACATTTGTTTTCACTTGCAAATGCAGCACCAAATGGCATGATTTTCTTTCACACGGTGCTATTACCAACAGCTTATG
AAACTGTGACGGCCTGGTCAGGTATTTGTGCGTCAGATGGCAATAGCACTTTTGGACTTGTTGTTAAGGATGTTTCAGCTGACG
CTATTTTCGCAATTTAGATGACAAATTCTACTTGACTCCTCGAACTATGTATCAGCCCAGAGTTGCAACTAGTTCTGATTTTGT
TCGAATAGAAGGTTGTGATGTGTTGTTTGTCAATGCAACTGTAATTGACTTGCCCTAGTATTATACCTGACTATATTGATATTA
ATCAAACCTGTTTCAGGACATATTAGAAAATTTTAGACCAAATTGGACTGTACCTGAGTTGACACTTGATATTTTTTAACGCAACC
TACTTAAACCTGACTGGTGAAATTAATGACTTAGAATTTAGGTCAGAGAAGCTACATAATACCACGGTAGAACTTGCTGTTCT
TATTGACAATATTAATAATACATTAGTCAATCTTGAATGGCTCAATAGAATT

CoV Hyena 34/07 (CoV Hyenas 07)

TAACCAGACTAGCAGAGGTTAGTGCTAGTAGACAACCTTGCTAAAGACAAGGTTAATGAATGCGTTAGGTCTCAATCCAGAGA
TTTGGATTCTGTGGTAATGGTACACATTTGTTTTCACTTGCAAATGCAGCACCAAATGGCATGATTTTCTTTCACACGGTGCT
ATTACCAACAGCTTATGAAACTGTGACGGCCTGGTCAGGTATTTGTGCGTCAGATGGCAATAGCACTTTTGGACTTGTTGTTA
AGGATGTTTCAGCTGACGCTATTTTCGCAATTTAGATGACAAATTCTACTTGACTCCTCGAACTATGTATCAGCCCAGAGTTGCA

ACTAGTTCTGATTTTGTTCGAATAGAAGGTTGTGATGTGTTGTTTGTCAATGCAACTGTAATTGACTTGCCTAGTATTATACC
TGACTATATTGATATTAATCAAACCTGTTTCAGGACATATTAGAAAATTTTAGACCAAATTGGACTGTACCTGAGTTGACACTTG
ATATTTTAAACGCAACCTACTTAAACCTGACTGGTGAAATTAATGACTTAGAATTTAGGTCAGAGAAGCTACATAATACCACG
GTAGAACTTGCTGTTCTTATTGACAATATTAATAATACATTAGTCAATCTTGAA

CoV Hyena 35/07 (CoV Hyenas 07)

TAACCAGACTAGCAGAGGTTAGTGCTAGTAGACAACCTGCTAAAGACAAGGTTAATGAATGCGTTAGGTCTCAATCCCAGAGA
TTTGGATTCTGTGGTAATGGTACACATTTGTTTTCACTTGCAAATGCAGCACCAAATGGCATGATTTTCTTTCACACGGTGCT
ATTACCAACAGCTTATGAAACTGTGACGGCCTGGTCAGGTATTTGTGCGTCAGATGGCAATAGCACTTTTGGAAGTTGTTGTTA
AGGATGTTTCAGCTGACGCTATTTTCGCAATTTAGATGACAAATTTCTACTTGACTCCTCGAACTATGTATCAGCCCAGAGTTGCA
ACTAGTTCTGATTTTGTTCGAATAGAAGGTTGTGATGTGTTGTTTGTCAATGCAACTGTAATTGACTTGCCTAGTATTATACC
TGACTATATTGATATTAATCAAACCTGTTTCAGGACATATTAGAAAATTTTAGACCAAATTGGACTGTACCTGAGTTGACACTTG
ATATTTTAAACGCAACCTACTTAAACCTGACTGGTGAAATTAATGACTTAGAATTTAGGTCAGAGAAGCTACATAATACCACG
GTAGAACTTGCTGTTCTTATTGACAATATTAATAATACATTAGTCAATCTTGAATG

CoV Hyena 36/07 (CoV Hyenas 07)

TAACCAGACTAGCAGAGGTTAGTGCTAGTAGACAACCTGCTAAAGACAAGGTTAATGAATGCGTTAGGTCTCAATCCCAGAGA
TTTGGATTCTGTGGTAATGGTACACATTTGTTTTCACTTGCAAATGCAGCACCAAATGGCATGATTTTCTTTCACACGGTGCT
ATTACCAACAGCTTATGAAACTGTGACGGCCTGGTCAGGTATTTGTGCGTCAGATGGCAATAGCACTTTTGGAAGTTGTTGTTA
AGGATGTTTCAGCTGACGCTATTTTCGCAATTTAGATGACAAATTTCTACTTGACTCCTCGAACTATGTATCAGCCCAGAGTTGCA
ACTAGTTCTGATTTTGTTCGAATAGAAGGTTGTGATGTGTTGTTTGTCAATGCAACTGTAATTGACTTGCCTAGTATTATACC
TGACTATATTGATATTAATCAAACCTGTTTCAGGACATATTAGAAAATTTTAGACCAAATTGGACTGTACCTGAGTTGACACTTG
ATATTTTAAACGCAACCTACTTAAACCTGACTGGTGAAATTAATGACTTAGAATTTAGGTCAGAGAAGCTACATAATACCACG
GTAGAACTTGCTGTTCTTATTGACAATATTAATAATACATTAGTCAATCTTGAA

CoV Hyena 37/07 (CoV Hyenas 07)

ACCAGACTAGCAGAGGTTAGTGCTAGTAGACAACCTGCTAAAGACAAGGTTAATGAATGCGTTAGGTCTCAATCCCAGAGATT
TGGATTCTGTGGTAATGGTACACATTTGTTTTCACTTGCAAATGCAGCACCAAATGGCATGATTTTCTTTCACACGGTGCTAT
TACCAACAGCTTATGAAACTGTGACGGCCTGGTCAGGTATTTGTGCGTCAGATGGCAATAGCACTTTTGGAAGTTGTTGTTAAG
GATGTTTCAGCTGACGCTATTTTCGCAATTTAGATGACAAATTTCTACTTGACTCCTCGAACTATGTATCAGCCCAGAGTTGCAAC
TAGTTCTGATTTTGTTCGAATAGAAGGTTGTGATGTGTTGTTTGTCAATGCAACTGTAATTGACTTGCCTAGTATTATACCTG
ACTATATTGATATTAATCAAACCTGTTTCAGGACATATTAGAAAATTTTAGACCAAATTGGACTGTACCTGAGTTGACACTTGAT
ATTTTAAACGCAACCTACTTAAACCTGACTGGTGAAATTAATGACTTAGAATTTAGGTCAGAGAAGCTACATAATACCACGGT
AGAACTTGCTGTTCTTATTGACAATATTAATAATACATTAGTCAATCTTGAATGGCTCAATAGAATT

CoV Hyena 44/07 (CoV Hyenas 07)

ACCAGACTAGCAGAGGTTAGTGCTAGTAGACAACCTGCTAAAGACAAGGTTAATGAATGCGTTAGGTCTCAATCCCAGAGATT
TGGATTCTGTGGTAATGGTACACATTTGTTTTCACTTGCAAATGCAGCACCAAATGGCATGATTTTCTTTCACACGGTGCTAT
TACCAACAGCTTATGAAACTGTGACGGCCTGGTCAGGTATTTGTGCGTCAGATGGCAATAGCACTTTTGGAAGTTGTTGTTAAG
GATGTTTCAGCTGACGCTATTTTCGCAATTTAGATGACAAATTTCTACTTGACTCCTCGAACTATGTATCAGCCCAGAGTTGCAAC
TAGTTCTGATTTTGTTCGAATAGAAGGTTGTGATGTGTTGTTTGTCAATGCAACTGTAATTGACTTGCCTAGTATTATACCTG
ACTATATTGATATTAATCAAACCTGTTTCAGGACATATTAGAAAATTTTAGACCAAATTGGACTGTACCTGAGTTGACACTTGAT
ATTTTAAACGCAACCTACTTAAACCTGACTGGTGAAATTAATGACTTAGAATTTAGGTCAGAGAAGCTACATAATACCACGGT
AGAACTTGCTGTTCTTATTGACAATATTAATAATACATTAGTCAATCTTGAATGG

CoV Hyena 62/07 (CoV Hyenas 07)

TAACCAGACTAGCAGAGGTTAGTGCTAGTAGACAACCTGCTAAAGACAAGGTTAATGAATGCGTTAGGTCTCAATCCCAGAGA
TTTGGATTCTGTGGTAATGGTACACATTTGTTTTCACTTGCAAATGCAGCACCAAATGGCATGATTTTCTTTCACACGGTGCT

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AGGATGTTTCAGCTGACGCTATTTTCGCAATTTAGATGACAAATTCTACTTGACTCCTCGAACTATGTATCAGCCCAGAGTTGCA
ACTAGTTCTGATTTTGTTCGAATAGAAGGTTGTGATGTGTTGTTTGTCAATGCAACTGTAATTGACTTGCCTAGTATTATACC
TGACTATATTGATATTAATCAAACCTGTTTCAGGACATATTAGAAAATTTTAGACCAAATTGGACTGTACCTGAGTTGACACTTG
ATATTTTAAACGCAACCTACTTAAACCTGACTGGTGAAATTAATGACTTAGAATTTAGGTCAGAGAAGCTACATAATACCACG
GTAGAACTTGCTGTTCTTATTGACAATATTAATAATACATTAGTCAATCTTGAA

CoV Hyena 82/07 (CoV Hyenas 07)

TCTCAGACTTTAACCAGACTAGCAGAGGTTAGTGCTAGTAGACAACCTGCTAAAGACAAGGTTAATGAATGCGTTAGGTCTCA
ATCCCAGAGATTTGGATTCTGTGGTAATGGTACACATTTGTTTTCACTTGCAAATGCAGCACCAAATGGCATGATTTTCTTTC
ACACGGTGCTATTACCAACAGCTTATGAACTGTGACGGCCTGGTCAGGTATTTGTGCGTCAGATGGCAATAGCACTTTTGGAA
CTTGTTGTTAAGGATGTTTCAGCTGACGCTATTTTCGCAATTTAGATGACAAATTCTACTTGACTCCTCGAACTATGTATCAGCC
CAGAGTTGCAACTAGTTCTGATTTTGTTCGAATAGAAGGTTGTGATGTGTTGTTTGTCAATGCAACTGTAATTGACTTGCCTA
GTATTATACCTGACTATATTGATATTAATCAAACCTGTTTCAGGACATATTAGAAAATTTTAGACCAAATTGGACTGTACCTGAG
TTGACACTTGATATTTTAAACGCAACCTACTTAAACCTGACTGGTGAAATTAATGACTTAGAATTTAGGTCAGAGAAGCTACA
TAATACCACGGTAGAACTTGCTGTTCTTATTGACAATATTAATAATACATTAGTCAATCTTGAATGG

CoV Hyena 91/07 (CoV Hyenas 07)

GTTAGTGCTAGTAGACAACCTGCTAAAGACAAGGTTAATGAATGCGTTAGGTCTCAATCCCAGAGATTTGGATTCTGTGGTAA
TGGTACACATTTGTTTTCACTTGCAAATGCAGCACCAAATGGCATGATTTTCTTTCACACGGTGCTATTACCAACAGCTTATG
AAACTGTGACGGCCTGGTCAGGTATTTGTGCGTCAGATGGCAATAGCACTTTTGGACTTGTGTTAAGGATGTTTCAGCTGACG
CTATTTTCGCAATTTAGATGACAAATTCTACTTGACTCCTCGAACTATGTATCAGCCCAGAGTTGCAACTAGTTCTGATTTTGT
TCGAATAGAAGGTTGTGATGTGTTGTTTGTCAATGCAACTGTAATTGACTTGCCTAGTATTATACCTGACTATATTGATATTA
ATCAAACCTGTTTCAGGACATATTAGAAAATTTTAGACCAAATTGGACTGTACCTGAGTTGACACTTGATATTTTAAACGCAACC
TACTTAAACCTGACTGGTGAAATTAATGACTTAGAATTTAGGTCAGAGAAGCTACATAATACCACGGTAGAACTTGCTGTTCT
TATTGACAATATTAATAATACATTAGTCAATCTTGAA

CoV Hyena 110/07 (CoV Hyenas 07)

GTTAGTGCTAGTAGACAACCTGCTAAAGACAAGGTTAATGAATGCGTTAGGTCTCAATCCCAGAGATTTGGATTCTGTGGTAA
TGGTACACATTTGTTTTCACTTGCAAATGCAGCACCAAATGGCATGATTTTCTTTCACACGGTGCTATTACCAACAGCTTATG
AAACTGTGACGGCCTGGTCAGGTATTTGTGCGTCAGATGGCAATAGCACTTTTGGACTTGTGTTAAGGATGTTTCAGCTGACG
CTATTTTCGCAATTTAGATGACAAATTCTACTTGACTCCTCGAACTATGTATCAGCCCAGAGTTGCAACTAGTTCTGATTTTGT
TCGAATAGAAGGTTGTGATGTGTTGTTTGTCAATGCAACTGTAATTGACTTGCCTAGTATTATACCTGACTATATTGATATTA
ATCAAACCTGTTTCAGGACATATTAGAAAATTTTAGACCAAATTGGACTGTACCTGAGTTGACACTTGATATTTTAAACGCAACC
TACTTAAACCTGACTGGTGAAATTAATGACTTAGAATTTAGGTCAGAGAAGCTACATAATACCACGGTAGAACTTGCTGTTCT
TATTGACAATATTAATAATACATTAGTCAATCTTGAATGGCTCAATAGAATTGAAA

CoV Jackal/07

GTTAGGGCTAGTAGACAACCTGCTAAAGACAAAGTTAATGAATGCGTTAGGTCTCAATCCCAAGATTTGGATTCTGTGGTAA
TGGTACACATTTGTTTTCACTTGCAAATGCAGCACCAAATGGCATGATTTTCTTTCACACAGTGCTATTACCAACAGCTTATG
AAACTGTGACGGCCTGGTCAGGTATTTGTGCATCAGATGGCGATCGCACTTTTGGACTTGTGTTAAGATGTCCAGCTGACG
CTATTTTCGCAATTTAGATGACAAATTCTATTTGACTCCCAGAACTATGTATCAGCCTAGAGTTGCAACTAGTTCTGATTTTGT
TCAAATTGAAGGTTGCGATGTCTTGTGTTGTCAATGCAACTGTAATTGAGTTGCCTAGCATTATACCTGATTATATCGATATTA
ATCAGACTGTTCAAGACATATTAGAAAATTATAGACCAAATTGGACAGTACCTGAATTAACATTTGACATTTTCAATGCCACC
TACTTAAACCTGACTGGTGAAATTAATGACTTAGAATTCAGGTCAGAAAAGCTACATAACACCACGGTAGAACTTGCTGTTCT
CATTGACAATATTAACAATACATTAGTCAATCTTGAATG

C. Nucleotide sequences of caliciviral RdRp gene fragments

I388/031226/SNP

GCAGAATCCAGCAGTGACAGCCACATCCTTGTCATCCTAGCTGGTTTCTGTGAAGACACGCCTATAACATCAGCTGCGGTGT
CTTGCCCTCGGTTACACCAGCACGCGGTTACGTGAATGATGTTTTCTTCCTAACACGGTCAGGCTTGCCCTCAGGAATGCCATTT
ACGTCGCAGATTAACTCACTCAACCATATGATTTACTTTGCTGCTGCAGTGCTTAAGGCATACATGGATAAGGGTGTACCGTA
TGACGGTAATGTTTTTGACATCGAACTGTCTTTACC

P420/040219/SNP

GCAGAATCCAGCAGTGACAGCCACATCCTTGTCATCCTAGCTGGTTTCTGTGAAGATACGCCTATAACATCAGCTGCAGTGT
CTTGCCCTCGGTTACACCAGCACGCGGTTACGTGAATGATGTTTTCTTCCTAACACGGTCAGGCTTACCCTCAGGAATGCCATTT
ACATCGCAGATAAACTCACTCAACCATATGATTTACTTTGCTGCTGCAGTGCTTAAGGCATATATGGACAAAGGTGTACCCTA
TGACGGCAATGTGTTTGACATCGAACTGTCTTTACC

P418/040221/SNP

GCAGAATCCAGCAGTGACAGCCACATCCTTGTCATCCTAGCTGGTTTCTGTGAAGATACGCCTATAACATCAGCTGCAGTGT
CTTGCCCTCGGTTACACCAGCACGCGGTTACGTGAATGATGTTTTCTTCCTAACACGGTCAGGCTTACCCTCAGGAATGCCATTT
ACATCGCAGATAAACTCACTCAACCATATGATTTACTTTGCTGCTGCAGTGCTTAAGGCATATATGGACAAAGGTGTACCCTA
TGACGGCAATGTGTTTGACATCGAACTGTCTTTACC

P427/040621/SNP

GCAGAATCCAGCAGTGACGGCCACATCCTTGTCATCCTAGCTGGTTTCTGTGAAGACACGCCTATAACATCAGCTGCAGTGT
CTTGCCCTCGGTTACACCAGCACGCGGTTACGTGAATGATGTTTTCTTCCTAACACGGTCAGGCTTGCCCTCAGGAATGCCATTT
ACATCGCAGATCAACTCACTTAACCATATGATTTACTTCGCTGCTGCAGTGCTTAAGGCATACATGGACAAGGGGGTACCGTA
TGACGGTAATGTTTTTGACATCGAACTGTCTTTACC

P448/050712/SNP

GCAGAATCCAGCAGTGACAGCCACATCCTTGTCATCCTAGCTGGTTTCTGTGAAGATACGCCTATAACATCAGCTGCAGTGT
CTTGCCCTCGGTTACACCAGCACGCGGTTACGTGAATGATGTTTTCTTCCTAACACGGTCAGGCTTACCCTCAGGAATGCCATTT
ACATCGCAGATAAACTCACTCAACCATATGATTTACTTTGCTGCTGCAGTGCTTAAGGCATATATGGACAAAGGTGTACCCTA
TGACGGCAATGTGTTTGACATCGAGACTGTCTTTACC

M532/060206/SNP

GCAGAATCCAGCAGTGACAGCCACATCCTTGTCATCCTAGCTGGTTTCTGTGAAGACACGCCTATAACATCGGCTGCAGTGT
CTTGCCCTCGGTTACACCAGCACGCGGTTACGTGAATGATGTTTTCTTCCTAACACGGTCAGGCTTGCCCTCAGGAATGCCATTT
ACATCGCAGATCAACTCACTTAACCATATGATTTACTTCGCTGCTGCAGTGCTTAAGGCATACATGGACAAGGGGGTACCGTA
TGACGGTAATGTTTTTGACATCGAACTGTCTTTACC

I422/060215/SNP

GCAGAATCCAGCAGTGACAGCCACATCCTTGTCATCCTAGCTGGTTTCTGTGAAGACACGCCTATAACATCAGCTGCAGTGT
CTTGCCCTCGGTTACACCAGCACGCGGTTACGTGAATGATGTTTTCTTCCTAACACGGTCAGGCTTACCCTCAGGAATGCCATTT
ACATCGCAGATAAACTCACTCAATCATATGATTTACTTTGCTGCTGCAGTGCTTAAGCATATATGGACAAAGGTGTACCCTA
TGACGGCAATGTGTTTGACATCGAACTGTCTTTACC

M425/020717/SNP

AGTGACAGCCACATCCTTGTCCATCCTAGCTGGTTTCTGTGAAGACACGCCTATAACATCAGCTGCAGTGTCTTGCCTCGGT
CACCAGCACGCGGTACGTGAATGATGTTTTCTTCTTAACACGGTCAGGCTTACCCTCAGGAATGCCATTTACATCGCAGATA
AACTCACTCAATCATATGATTTACTTTGCTGCTGCAGTGCT

M435/021229/SNP

AGTGACAGCCACATCCTTGTCCATCCTAGCTGGTTTCTGTGAAGACACGCCTATAACATCAGCTGCGGTGTCTTGCCTCGGT
CACCAGCACGCGGTACGTGAATGATGTTTTCTTCTTAACACGGTCAGGCTTGCCTTCAGGAATGCCATTTACGTGCGAGATT
AACTCACTCAACCATATGATTTACTTTGCTGCTGCAGTGCT

E085/021219/NC

CAGTGACAGCCACATCCTTGTCCATCCTAGCTGGTTTCTGTGAAGACACGCCTATAACATCAGCTGCAGTGTCTTGCCTCGGT
TCACCAGCACGCGGTACGTGAATGATGTTTTCTTCTTAACACGGTCAGGCTTACCCTCAGGAATGCCATTTACATCGCAGAT
AAACTCACTCAATCATATGATTTACTTTGCTGCTGCAGTGCT

A056/030811/NC

AGTGACAGCCACATCCTTGTCCATCCTAGCTGGTTTCTGTGAAGACACGCCTATAACATCAGCTGCAGTGTCTTGCCTCGGT
CACCAGCACGCGGTACGTGAATGATGTTTTCTTCTTAACACGGTCAGGCTTACCCTCAGGAATGCCATTTACATCGCAGATA
AACTCACTCAATCATATGATTTACTTTGCTGCTGCAGTGCT

Z118_lu/040825/SNP

CAGTGACAGCCACATCCTTGTCCATCCTAGCTGGTTTCTGTGAAGACACGCCTATAACATCAGCTGCAGTGTCTTGCCTCGGT
TCACCAGCACGCGGTACGTGAATGATGTTTTCTTCTTAACACGGTCAGGCTTACCCTCAGGAATGCCATTTACATCGCAGAT
AAACTCACTCAACCATATGATTTACTTTGCTGCTGCAGTGCT

I403/070507/SNP

CAGTGACAGCCACATCCTTGTCCATCCTAGCTGGTTTCTGTGAAGACACGCCTATAACATCAGCTGCAGTGTCTTGCCTCGGT
TCACCAGCACGCGGTACGTGAATGATGTTTTCTTCTTAACACGGTCAGGCTTACCCTCAGGAATGCCATTTACATCGCAGAT
AAACTCACTCAATCATATGATTTACTTTGCTGCTGCAGTGC

P412/071001/SNP

CAGTGACAGCCACATCCTTGTCCATCCTAGCTGGCTTTTGTGAAGACACACCCATAACATCTGCTGCGGTGTCCTGTCTTGGT
TCACCAGCGCGTGGTTATGTGAATGATGTTTTCTTCTTAACACGGTCTGGCCTCCCTTCGGGCATGCCGTTACCTCACAGAT
AAATTCACTCAACCACATGATTTACTTTGCTGCTGCAGTGC

I459/071108/SNP

CAGTGACAGCCACATCCTTGTCCATCCTAGCTGGTTTCTGTGAAGACACGCCTATAACATCAGCTGCAGTGTCTTGCCTCGGT
TCACCAGCACGCGGTACGTGAATGATGTTTTCTTCTTAACACGGTCAGGCTTACCCTCAGGAATGCCATTTACATCGCAGAT
AAACTCACTCAATCATATGATTTACTTTGCTGCTGCAGTGC

D. Nucleotide sequences of *Hepatozoon* sp. 18S rRNA fragments

HY I392 SNP (HY SNP)

AGTCATATGCTTGTCTTAAAGATTAAGCCATGCATGTCTAAGTATAAACAGTAATACAGTAAAACTGCAAATGGCTCATTAAA
ACAGTTATAGTTTACTTGATAATAAGTTTTTACATGGATAACCGTGGTAATTCTAGAGCTAATACATGAGCAAAATCTCAACT
TTTTTAGGAGAGATGCATTTATTAGATAAAAAATCAATACATGCTTTTAAAGTATGGAATTTGGTGAATTACAGTAACCTAGC
AAATCGCATAGTGAAAACAGGCGATAAATCATTCAAGTTTCTGACCTATCAGCTTTCGACGGTATGGTATTGGCTTACCGTGG
CAGTGACGGTTAACGGGGGATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGG
CGCGCAAATTACCCAATTCTAACAGCATAAGAGAGGTAGTGACAAGAAATAACAATACAAGGCAGTTAAAAATGCTTTGTAATT
GGAATGATAGAAATTTAAACACTTTTTTAAAGTATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGTAATTCCAGCTCCA
ATAGCGTATATTAAAAATTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTCTGCTAAAAATAACCGGTCTGCTTTTAATAAAGGT
GGTATCTTGGTGTGTTTTTAGCAATAATGTCCTTTGAAATATTTTTTACTTCATTGTAATAAATTATGTTTCAGGATTTT

HY I299 SNP (HY SNP)

AGTCATATGCTTGTCTTAAAGATTAAGCCATGCATGTCTAAGTATAAACAGTAATACAGTAAAACTGCAAATGGCTCATTAAA
ACAGTTATAGTTTACTTGATAATAAGTTTTTACATGGATAACCGTGGTAATTCTAGAGCTAATACATGAGCAAAATCTCAACT
TTTTTAGGAGAGATGCATTTATTAGATAAAAAATCAATACATGCTTTTAAAGTATGGAATTTGGTGAATTACAGTAACCTAGC
AAATCGCATAGTGAAAACAGGCGATAAATCATTCAAGTTTCTGACCTATCAGCTTTCGACGGTATGGTATTGGCTTACCGTGG
CAGTGACGGTTAACGGGGGATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGG
CGCGCAAATTACCCAATTCTAACAGCATAAGAGAGGTAGTGACAAGAAATAACAATACAAGGCAGTTAAAAATGCTTTGTAATT
GGAATGATAGAAATTTAAACACTTTTTTAAAGTATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGTAATTCCAGCTCCA
ATAGCGTATATTAAAAATTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTCTGCTAAAAATAACCGGTCTGCTTTTAATAAAGGT
GGTATCTTGGTGTGTTTTTAGCAATAATGTCCTTTGAAATATTTTTTACTTCATTGTAATAAATTATGTTTCAGGATTTT

M183 SNP (HY SNP)

AGTCATATGCTTGTCTTAAAGATTAAGCCATGCATGTCTAAGTATAAACAGTAATACAGTAAAACTGCAAATGGCTCATTAAA
ACAGTTATAGTTTACTTGATAATAAGTTTTTACATGGATAACCGTGGTAATTCTAGAGCTAATACATGAGCAAAATCTCAACT
TTTTTAGGAGAGATGCATTTATTAGATAAAAAATCAATACATGCTTTTAAAGTATGGAATTTGGTGAATTACAGTAACCTAGC
AAATCGCATAGTGAAAACAGGCGATAAATCATTCAAGTTTCTGACCTATCAGCTTTCGACGGTATGGTATTGGCTTACCGTGG
CAGTGACGGTTAACGGGGGATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGG
CGCGCAAATTACCCAATTCTAACAGCATAAGAGAGGTAGTGACAAGAAATAACAATACAAGGCAGTTAAAAATGCTTTGTAATT
GGAATGATAGAAATTTAAACACTTTTTTAAAGTATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGTAATTCCAGCTCCA
ATAGCGTATATTAAAAATTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTCTGCTAAAAATAACCGGTCTGCTTTTAATAAAGGT
GGTATCTTGGTGTGTTTTTAGCAATAATGTCCTTTGAAATATTTTTTACTTCATTGTAATAAATTATGTTTCAGGATTTT

P193 SNP (HY SNP)

AGTCATATGCTTGTCTTAAAGATTAAGCCATGCATGTCTAAGTATAAACAGTAATACAGTAAAACTGCAAATGGCTCATTAAA
ACAGTTATAGTTTACTTGATAATAAGTTTTTACATGGATAACCGTGGTAATTCTAGAGCTAATACATGAGCAAAATCTCAACT
TTTTTAGGAGAGATGCATTTATTAGATAAAAAATCAATACATGCTTTTAAAGTATGGAATTTGGTGAATTACAGTAACCTAGC
AAATCGCATAGTGAAAACAGGCGATAAATCATTCAAGTTTCTGACCTATCAGCTTTCGACGGTATGGTATTGGCTTACCGTGG
CAGTGACGGTTAACGGGGGATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGG
CGCGCAAATTACCCAATTCTAACAGCATAAGAGAGGTAGTGACAAGAAATAACAATACAAGGCAGTTAAAAATGCTTTGTAATT
GGAATGATAGAAATTTAAACACTTTTTTAAAGTATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGTAATTCCAGCTCCA
ATAGCGTATATTAAAAATTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTCTGCTAAAAATAACCGGTCTGCTTTTAATAAAGGT
GGTATCTTGGTGTGTTTTTAGCAATAATGTCCTTTGAAATATTTTTTACTTCATTGTAATAAATTATGTTTCAGGATTTT

P451 SNP (HY SNP)

AGTCATATGCTTGTCTTAAAGATTAAGCCATGCATGTCTAAGTATAAACAGTAATACAGTAAAACTGCAAATGGCTCATTAAA
ACAGTTATAGTTTACTTGATAATAAGTTTTTACATGGATAACCGTGGTAATTCTAGAGCTAATACATGAGCAAAATCTCAACT
TTTTTAGGAGAGATGCATTTATTAGATAAAAAATCAATACATGCTTTTAAAGTATGGAATTTGGTGAATTACAGTAACCTAGC
AAATCGCATAGTGA AACAGGCGATAAATCATTCAAGTTTCTGACCTATCAGCTTTCGACGGTATGGTATTGGCTTACCGTGG
CAGTGACGGTTAACGGGGGATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGG
CGCGCAAATTACCCAATTCTAACAGCATAAGAGAGGTAGTGACAAGAAATAACAATACAAGGCAGTTAAATGCTTTGTAATT
GGAATGATAGAAATTTAAACACTTTTTTAAAGTATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGTAATTCCAGCTCCA
ATAGCGTATATTAAATTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTCTGCTAAAAATAACCGGTCTGCTTTTAATAAAGGT
GGTATCTTGGTGTGTTTTTAGCAATAATGTCCTTTGAAATATTTTTTACTTCATTGTAATAAATTATGTTTCAGGATTTT

HY Z118 SNP (HY SNP)

AGTCATATGCTTGTCTTAAAGATTAAGCCATGCATGTCTAAGTATAAACAGTAATACAGTAAAACTGCAAATGGCTCATTAAA
ACAGTTATAGTTTACTTGATAATAAGTTTTTACATGGATAACCGTGGTAATTCTAGAGCTAATACATGAGCAAAATCTCAACT
TTTTTAGGAGAGATGCATTTATTAGATAAAAAATCAATACATGCTTTTAAAGTATGGAATTTGGTGAATTACAGTAACCTAGC
AAATCGCATAGTGA AACAGGCGATAAATCATTCAAGTTTCTGACCTATCAGCTTTCGACGGTATGGTATTGGCTTACCGTGG
CAGTGACGGTTAACGGGGGATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGG
CGCGCAAATTACCCAATTCTAACAGCATAAGAGAGGTAGTGACAAGAAATAACAATACAAGGCAGTTAAATGCTTTGTAATT
GGAATGATAGAAATTTAAACACTTTTTTAAAGTATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGTAATTCCAGCTCCA
ATAGCGTATATTAAATTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTCTGCTAAAAATAACCGGTCTGCTTTTAATAAAGGT
GGTATCTTGGTGTGTTTTTAGCAATAATGTCCTTTGAAATATTTTTTACTTCATTGTAATAAATTATGTTTCAGGATTTT

Z122 SNP (HY SNP)

AGTCATATGCTTGTCTTAAAGATTAAGCCATGCATGTCTAAGTATAAACAGTAATACAGTAAAACTGCAAATGGCTCATTAAA
ACAGTTATAGTTTACTTGATAATAAGTTTTTACATGGATAACCGTGGTAATTCTAGAGCTAATACATGAGCAAAATCTCAACT
TTTTTAGGAGAGATGCATTTATTAGATAAAAAATCAATACATGCTTTTAAAGTATGGAATTTGGTGAATTACAGTAACCTAGC
AAATCGCATAGTGA AACAGGCGATAAATCATTCAAGTTTCTGACCTATCAGCTTTCGACGGTATGGTATTGGCTTACCGTGG
CAGTGACGGTTAACGGGGGATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGG
CGCGCAAATTACCCAATTCTAACAGCATAAGAGAGGTAGTGACAAGAAATAACAATACAAGGCAGTTAAATGCTTTGTAATT
GGAATGATAGAAATTTAAACACTTTTTTAAAGTATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGTAATTCCAGCTCCA
ATAGCGTATATTAAATTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTCTGCTAAAAATAACCGGTCTGCTTTTAATAAAGGT
GGTATCTTGGTGTGTTTTTAGCAATAATGTCCTTTGAAATATTTTTTACTTCATTGTAATAAATTATGTTTCAGGATTTT

HY P162 SNP

AGTCATATGCTTGTCTTAAAGATTAAGCCATGCATGTCTAAGTATAAACAGTAATACAGTAAAACTGCAAATGGCTCATTAAA
ACAGTTATAGTTTACTTGATAATAAGTTTTTACATGGATAACCGTGGTAATTCTAGAGCTAATACATGAGCAAAATCTCAACT
TTTTTAGGGGAGATGCATTTATTAGATAAAAAATCAATACATGCTTTTAAAGTATGGAATTTGGTGAATTACAATAACATAGC
AAATCGCATAGTGA AACAGGCGATAAATCATTCAAGTTTCTGACCTATCAGCTTTCGACGGTATGGTATTGGCTTACCGTGG
CAGTGACGGTTAACGGGGGATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGG
CGCGCAAATTACCCAATTCTAACAGCATAAGAGAGGTAGTGACAAGAAATAACAATACAAGGCAGTTAAATGCTTTGTAATT
GGAATGATAGAAATTTAAAGACTTTTTTAAAGTATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGTAATTCCAGCTCCA
ATAGCGTATATTAAATTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTCTGCTAAAAATAACCGGTCTGCTTTTAATAAAGGT
GGTATCTTGGTGTGTTTTTAGCAATAATGTCCTTTGAAATGTTTTTTACTTCATTGTAATAAATTATATTGAGGATTTT

HY P279 SNP

AGTCATATGCTTGTCTTAAAGATTAAGCCATGCATGTCTAAGTATAAACAGTAATACAGTAAAACTGCAAATGGCTCATTAAA
ACAGTTATAGTTTACTTGATAATAAGTTTTTACATGGATAACCGTGGTAATTCTAGAGCTAATACATGAGCAAAATCTCAACT

TTTTTAGGAGAGATGCATTTATTAGATAAAAAATCAATACATGCTTTTAAAGTATGGAATTTGGTGAATTACAGTAACTTAGC
AAATCGCATAGTGAAAACAGGCGATAAATCATTCAAGTTTCTGACCTATCAGCTTTCGACGGTATGGTATTGGCTTACCGTGG
CAGTGACGGTTAACGGGGGATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGG
CGCGCAAATTACCCAATTCTAACAGCATAAGAGAGGTAGTGACAAGAAATAACAATACAAGGCAGTTAAATGCTTTGTAATT
GGAATGATAGAAATTTAAACACTTTTTAAAGTATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCA
ATAGCGTATATTAAAAATTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTCTGCTAAAAATAACCGGTCTGCTTTTAATAAAGGT
GGTATCTTGGTGTGTTTTAAGCAATAATGTCCTTTGAAATATTTTTACTTTCATTGTAATAAATTATGTTTCAGGATTTT

HY Z94 SNP

AGTCATATGCTTGTCTTAAAGATTAAGCCATGCATGTCTAAGTATAAACAGTAATACAGTAAAACTGCAAATGGCTCATTAAA
ACAGTTATAGTTTACTTGATAATAAGTTTTTACATGGATAACCGTGGTAATTCTAGAGCTAATACATGAGCAAAATCTCAACT
TTTTTAGGAGAGATGCATTTATTAGATAAAAAATCAATACATGCTTTTAAAGTATGGAATTTGGTGAATTACAATAACTTAGC
AAATCGCATAGTGAAAACAGGCGATAAATCATTCAAGTTTCTGACCTATCAGCTTTCGACGGTATGGTATTGGCTTACCGTGG
CAGTGACGGTTAACGGGGGATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGG
CGCGCAAATTACCCAATTCTAACAGCATAAGAGAGGTAGTGACAAGAAATAACAATACAAGGCAGTTAAATGCTTTGTAATT
GGAATGATAGAAATTTAAACACTTTTTAAAGTATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCA
ATAGCGTATATTAAAAATTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTCTGCTAAAAATAACCGGTCTGCTTTTAATAAAGGT
GGTATCTTGGTGTGTTTTAGCAATAATGTCCTTTGAAATGTTTTTTACTTTCATTGGAATAAATTATATTCAGGATTTT

FX23 SNP

AGTCATATGCTTGTCTTAAAGATTAAGCCATGCATGTCTAAGTATAAACAGTAATACAGTAAAACTGCAAATGGCTCATTAAA
ACAGTTATAGTTTACTTGATAATAAGTTTTTACATGGATAACCGTGGTAATTCTAGAGCTAATACATGAGCAAAATCTTAACT
TTTTTAGGAGAGATGGATTTATTAGATAAAAAATCAATACATGCTTTTAAAGTATGGAATTTGGTGAATTACAATAACTTAGC
AAATCGCATAGTGAAAACAGGCGATAAATCATTCAAGTTTCTGACCTATCAGCTTTCGACGGTATGGTATTGGCTTACCGTGG
CAGTGACGGTTAACGGGGGATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGG
CGCGCAAATTACCCAATTCTAACAGCATAAGAGAGGTAGTGACAAGAAATAACAATACAAGGCAGTTAAATGCTTTGTAATT
GGAATGATAGAAATTTAAACACTTTTTAAAGTATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCA
ATAGCGTATATTAAAAATTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTCTGCTAAAAGTAACCGGTCTGCTTTTAATAAAGGT
GGTATCTTGGTGTGTTTTAGCAATAATGTCCTTTGAAATGTTTTTTACTTTCATTGTAATAAATTATATTCAGGATTTT

WTM14 SNP

TGNNANATCCTTGTNNTAAAGATTAACCCATGCATGTCTAAGTATCAACCATAATACGGTAAAACTGCAAATGGCTCATTAAA
ACAGTTATAGTTTATTTGATAATAAAATTTTACATGGATTACCGTGGTAATTTTAGAGTTAATACATGAGCAAAATTTCACTTT
TTTTAGAAGAGATGCATTTATTAGATAAAAAACCAATACATGCTTTTCAAGTATGAAATTTGGTGATTTACAATAACTTAGCA
AATCGCATAGTGAAAACGGCGATAAATCATTCAAGTTTCTGACCTATCAGCTTTCGACGGTATGGTATTGGCTTACCGTGGC
AGTGACGGTTAACGGGGGATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGGC
GCGCAAATTACCCAATTCTAACAGCATAAGAGAGGTAGTGACAAGAAATAACAATACAAGGCAGTTAAATGCTTTGTAATTG
GAATGATAGAAATTTAAACACTTTTTAAAGTATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAA
TAGCGTATATTAAAAATTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTCTGTTAAAAATAACCGGTCTGCTTTTATTAAGGT
GTATCTTGGTGTGTTTTAGCAATAATGTCCTTTGAAATGTTTTTTACTTTATTGTAATAAATAATATTCAGGATTTT

GE16 SNP

AGTCATATGCTTGTCTTAAAGATTAAGCCATGCATGTCTAAGTATAAACAGTAATACAGTAAAACTGCAAATGGCTCATTAAA
ACAGTTATAGTTTACTTGATAATAAAATTTTACATGGATAACCGTGGTAATTCTAGAGCTAATACATGAGCAAAATCTCAACT
TTTTTAGAAGAGATGCATTTATTAGATAAAAAACCAATGCATGCTTTTGAAGTATGAAATTTGGTGATTTACAATAACTTAGC
AAATCGCATAGTGAAAACAGGCGATAAATCATTCAAGTTTCTGACCTATCAGCTTTCGACGGTATGGTATTGGCTTACCGTGG
CAGTGACGGTTAACGGGGGATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGG
CGCGCAAATTACCCAATTCTAACAGCATAAGAGAGGTAGTGACAAGAAATAACAATACAAGGCAGTTAAATGCTTTGTAATT

GGAATGATAGAAATTTAAACACTTTTTAAAGTATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGTAATTCAGCTCCA
ATAGCGTATATTAAATTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTCTGCTAAAAATTAACCGGTCTGCTTTTAATAAAGGT
GGTATCTTGGTGTGTTTTTAGCAATAATGTCCTTTGAAATGTTTTTTACTTCATTGTAATTAATATATTCAGGATTTT

LI 01 SNP

AGTCATATGCTTGTCTTAAAGATTAAGCCATGCATGTCTAAGTATAAACAGTAATACAGTAAACCTGCAAATGGCTCATTAAA
ACAGTTATAGTTTACTTGATAATAAGTTTTTACATGGATAACCGTGGTAATTCTAGAGCTAATACATGAGCAAAATCTTAACT
TTTTTAGGAGAGATGCATTTATTAGATAAAAAATCAATACATGCTTTTAAAGTATGGAATTTGGTGAATTACAATAACTTAGC
AAATCGCATAGTAAAAACAGGCGATAAATCATTCAAGTTTCTGACCTATCAGCTTTCGACGGTATGGTATTGGCTTACCGTGG
CAGTGACGGTTAACGGGGGATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGG
CGCGCAAATTACCCAATTCTAACAGCATAAGAGAGGTAGTGACAAGAAAATAACAATACAAGGCAGTTAAAAATGCTTTGTAATT
GGAATGATAGAAATTTAAACACTTTTTAAAGTATCAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGTAATTCAGCTCCA
ATAGCGTATATTAAATTTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTCTGCTAAAAATAACCGGTCTGCTTTTAATAAAGGT
GGTATCTTGGTGTGTTTTTAGCAATAATGTCCTTTGAAATGTTTTTTACTTCATTGTAATAAATTATATTCAGGATTTT

AWD Lpi6499 LGCA

TGCATGTCTAAGTATAAGCAAAAATACAGCAAACTGCAAATGGCTCATTAACAGTTATGGTTTACTTGATAATAAATTTT
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CTTTGAAGTGTTTTTTACTTTTATTGTAATAAAGCATATTCAGGGCTTT

AWD Lpi6514 LGCA

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HY A84 NC

AGTCATATGCTTGTCTTAAAGATTAAGCCATGCATGTCTAAGTATAAACAGTAATACAGTAAACTGCAAATGGCTCATTAAA
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CGCGCAAATTACCCAATTCTAACAGCATAAGAGAGGTAGTGACAAGAAAATAACAATACAAGGCAGTTAAAAATGCTTTGTAATT
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HY A163 NC

AGTCATATGCTTGTCTTAAAGATTAAGCCATGCATGTCTAAGTATAAACAGTAATACAGTAAAACTGCAAATGGCTCATTAAA
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HY X34 NC (HY NC)

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HY X47 NC (HY NC)

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CAGTGACGGTTAACGGGGGATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGG
CGCGCAAATTACCCAATTCTAACAGCATAAGAGAGGTAGTGACAAGAAATAACAATACAAGGCAGTTAAAAATGCTTTGTAATT
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ATAGCGTATATTAAAAATGTTGCAGTTAAAAAGCTCGTAGTTGAATTTCTGCTAAAAATAACCGATCTGCTTTTAATAAAGGT
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HY F42 NC

AGTCATATGCTTGTCTTAAAGATTAAGCCATGCATGTCTAAGTATAAACAGTAATACAGTAAAACTGCAAATGGCTCATTAAA
ACAGTTATAGTTTACTTGATAATAAGTTTTTACATGGATAACCGTGGTAATTCTAGAGCTAATACATGAGCAAAATCTCAACT
TTTTTAGGAGAGATGCATTTATTAGATAAAAAATCAATACATGCTTTTAAAGTATGGAATTTGGTGAATTACAGTAACCTAGC
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CAGTGACGGTTAACGGGGGATTAGGGTTCGATTCCGGAGAGGGAGCCTGAGAAACGGCTACCACATCTAAGGAAGGCAGCAGG
CGCGCAAATTACCCAATTCTAACAGCATAAGAGAGGTAGTGACAAGAAATAACAATACAAGGCAGTTAAAAATGCTTTGNAATT
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ATAGCGTATATTAAAAATGTTGCAGTTAAAAAGCTCGTAGTTGAATTTCTGCTAAAAATAACCGGTCTGCTTTTAATAAAGGT
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LI02 NC

AGTCATATGCTTGTTTTAAAGATTAAGCCATGCATGTCTAAGTATAAACAATAATACAGTAAAACTGCAAATGGCTCATTAAA
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TGGTATCTTGGTGTGTTTTTAGCAATAATGTCCTTTGAAATGTTTTTTACTTTATTGTAATAAATAATATTCAGGATTTT

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SELBSTSTÄNDIGKEITSERKLÄRUNG

Hiermit erkläre ich, dass ich die vorliegende Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet habe. Ich versichere, dass diese Arbeit in dieser oder anderer Form noch keiner anderen Prüfungsbehörde vorgelegt wurde.

Der Inhalt der Promotionsordnung der Mathematisch - Naturwissenschaftlichen Fakultät I der Humboldt Universität zu Berlin vom 01.10.2002 ist mir bekannt.

Greifswald,

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